The Chemical Dynamics of BONE MINERAL

By

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To

CAROL

PETE

and

RUSS

Preface

The physiology of bone is broad and complex. For example, clinical data from the study of disease states in man and X-ray diffraction data from the study of the physics of the solid state are equally pertinent to an understanding of the subject. No one can presume to be an expert in all the diverse disciplines that contribute to our growing knowledge of bone. In brief, probably no one individual can make a general synthesis of the subject that is accurate in all respects.

One can try, however, and this book is the record of such an attempt. The unifying concepts have been sought at the molecular level, and these, as building materials, have been assembled by physicochemical and biochemical rules and views. The resultant structure or general synthesis will in all probability not last long. In the final phases of construction, there was a severe shortage of good building material—established experimental results. By the substitution of unconfirmed preliminary findings and speculation for hard fact, the job was completed. With use, as new data arrive on the scene, many alterations will be necessary. The authors feel strongly, however, that such a structure, or general synthesis, is very much needed as a place where the various disciplines can meet, interact, and get along together. The authors also believe that the keystone and the foundation on which this book was built are fundamentally sound. The foundation is the surface chemistry of the mineral substance of bone. The keystone is the recognition of the peculiar solubility properties of this mineral substance. Bone mineral can and does exhibit widely varying solubilities. For this reason, a given concentration product or activity product Ca × P, in certain histological areas, can be considered undersaturated, in others just saturated, while in other areas, serum in particular, the same product represents a highly supersaturated condition. How this paradox comes about and its importance in the calcification process and homeostasis are explained in the following pages. It is on this point that many earlier attempts at a general synthesis of the problem

have failed Most of these were based on the assumption of a fixed K_{sp} for bone mineral Thus the common observation that *the serum product* $Ca \times P$ *is not constant* was left unexplained at the very start

The purpose of this monograph is to stimulate interest and research It is unimportant whether the reader is pleased pained or rendered righteously indignant The book will have failed its purpose only if it does not engender some kind of positive reaction in the reader the desire to prove to disprove or to know

The book is aimed at the inherently curious the clinician who wonders why the anatomist who wonders where the chemist who wonders how and just any investigator who wonders whether

It is with the greatest pleasure that the authors acknowledge their indebtedness to their colleagues, at the University of Rochester and other institutions who have kindly read and criticized the manuscript and otherwise given counsel and advice in the course of its preparation. They are especially indebted to Drs Gilbert Forbes, Harold C. Hodge John E Howard, Robert A. Robinson, and A. Raymond Terepka. It was Dr Franklin C. McLean however who inspired the authors to undertake the task of writing the monograph who rejuvenated their failing resolve and who by kindly advice and suggestion softened mistakes and strengthened weak passages throughout the development of the manuscript Thanks are due to many others to C L. Comar R Amprino and P Lacroix for unpublished illustrative material and to our colleagues of the Josiah Macy Jr and Gordon Research conferences for many inspiring discussions and pleasant associations

Mrs. Joyce Cole has earned the title of nurse and midwife in the birth of this monograph which incidentally required a gestation period of approximately nine months. She prepared all the illustrations typed and retyped the manuscript corrected our spelling, and minimized interruptions and diversions. For all of this and, above all, for her unfailingly co-operative good spirit, our heartfelt thanks and gratitude are here acknowledged.

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Contents

CHAPTER I

Solution Chemistry

For many many years chemists and biochemists have tried to decipher the puzzling interrelationships between blood and bone, for in this problem lies the key to an understanding of much of bone physiology and electrolyte metabolism. On first inspection the problem appears quite simple. In the living organism the skeleton contains a huge quantity of extracellular crystalline sparingly soluble mineral salts of calcium and phosphate. One would be inclined to reason that, at all times, the blood must be exactly saturated with respect to the bone mineral. Indeed there is much to suggest the operation of a simple solubility product. Normally the product of calcium and inorganic phosphorus in serum is quite constant. In many pathological states the levels of calcium and phosphate vary reciprocally. On the other hand there are also clinical conditions in which the serum levels of both ions may be elevated or depressed in apparent violation of the principle of a simple solubility product. Vitamin D and the secretion of the parathyroid gland have a profound influence on the circulating levels of calcium and phosphate. How can a vitamin and a hormone influence a solubility equilibrium?

The literature is bulging with reports of studies on the solubility of bone mineral in aqueous solution. Some of these reports suggest that serum must be regarded as a supersaturated solution of bone mineral others imply that serum is undersaturated. Thus the most fundamental question in bone physiology has been perceived but dimly shrouded in a veil of conflicting paradoxical data.

Part of this confusion was man-made, through the introduction of artifacts and errors in experimental techniques. Not all but many investigators failed to take into account the importance of ionic strength and specific interion attraction as sources of large error. Very few researchers took the trouble to think and plan in thermodynamic terms.

If we wish to understand the interrelationships between blood and bone, we must begin at the beginning. We must develop an understand

ing of modern concepts of solution chemistry, we must determine the correction factors necessary to an expression of the true or *effective concentrations* the chemical activities of calcium and inorganic phosphate in normal serum. In brief we must review the principles of physical chemistry and attempt to apply these principles to our physiological problem.

Non-specific Ion Effects

The history of physical chemistry (as with all sciences) can be well described as a progressive series of approximations. A general law is enunciated. Then aberrant departures from ideality are sought and observed and a new more precise, formulation is devised empirically Finally, the reasons for this exceptional behavior are recognized and a revised law is derived on theoretical grounds.

Such is the case with solution chemistry The first general law was Raoult s law relating the colligative properties of solutions (lowering of vapor pressure, rise in boiling point, depression of freezing point, and osmotic pressure) to the number of solute atoms or ions i.e. to the molecular concentration of the solute in the solution and not to the nature of the solute.

Apparent failure of the law was soon observed. In non-hydroxylic solvents many substances seemed to exert only half as much effect as expected indicating *association* while in aqueous solutions many substances showed several times as much effect as expected from Raoult s law indicating *dissociation*

Without explaining the discrepancy Van t Hoff expressed as a ratio the observed properties relative to those expected For salts such as NaCl KNO_3 or $MgSO_4$ in aqueous solution, this Van t Hoff ratio or factor i approached a limiting value of 2 0 while salts like K_2SO_4 $CaCl_2$ and $NaHSO_4$ gave a limiting ratio of 3 0

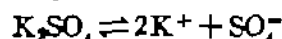
THE ARRHENIUS CONCEPT OF DISSOCIATION

Arrhenius recognized the fundamental significance of the Van t Hoff factor He suggested that when an acid, base, or salt is dissolved in water it splits up or dissociates into positively and negatively charged ions. This was the first statement of the theory of electrolytic dissociation. Thus a salt such as sodium chloride will dissociate into two ions



$$NaCl \rightleftharpoons Na^+ + Cl^-$$

whereas potassium sulfate yields three ions



$$K_2SO_4 \rightleftharpoons 2K^+ + SO_4^=$$

This was truly a great advance, but the problem was far from solved Only near infinite dilution did experiment agree with theory For many years a catch-all explanation was given salts do not dissociate completely except at infinite dilution The explanation is of course substantially correct for weak electrolytes. However we recognize today that strong monovalent electrolytes, such as the halides and nitrates of the alkali and alkali-earth cations are completely dissociated at all reasonable concentrations, even in the solid crystalline state. Some new explanation was required

CONCEPT OF ION ACTIVITIES

The true explanation for much of the departure from ideality was finally given by the interionic attraction theory The basis of this theory in simplest terms, is that in very dilute solutions, the individual ions are relatively far apart and can exert little influence on one another As the concentration is increased, however the attraction among the more closely packed ions of opposite charge decreases the physicochemical activity of these ions. This can be expressed in terms of mobility Every ion may be regarded as being surrounded by a centrally symmetric ionic atmosphere of charge opposite in sign to that of the ion itself If an electromotive force is applied the ion migrates away from its surrounding field, which in turn sets up an electrostatic field retarding its speed The tendency of the ionic atmosphere to move in a direction opposite to that of the central ion further retards its mobility

This, of course, is a qualitative and approximate description of the meaning of the concept of interionic attraction. The *magnitude* of interionic attraction depends on the closeness of the various charged ions (their concentration) and the magnitude of their charge (their valence) The term ionic strength " μ was introduced by Lewis and Randall (27) to describe the numbers and charges of ions in a given solution i.e. the intensity of the electrical field due to the ions present in the solution. Ionic strength μ, is defined as one half the sum of the terms obtained by multiplying the molality (or concentration) of each ionic species present in solution by the square of its valence, thus,

$$\mu = \tfrac{1}{2} \Sigma c_i z^2$$

where c is the actual concentration of each ion and z_i is its valence

It was readily apparent that the true or effective concentration expressed as chemical activity a, differed from the actual concentration c_i as a function of field strength μ. The correction factor needed to

convert concentration to true chemical potential or activity is termed the *activity coefficient* represented by the symbol f or γ. In strict usage, the activity coefficient must be rigorously defined. For present purposes, we shall assume, for a simple representative salt, say potassium chloride,

$$a_{KCl} = f_{KCl} \cdot c_{KCl} \quad \text{or} \quad f_{KCl} = \frac{a_{KCl}}{c_{KCl}}$$

where a_{KCl} is the true or effective concentration or activity, f_{KCl} is the mean activity coefficient of KCl (in the general case the mean activity coefficient of the positive and negative ions is written $f_{\pm}$) and c_{KCl} is the actual molarity of the KCl solution.

Debye and Hückel are primarily responsible for placing the theory of interionic attraction on a quantitative basis. They derived equations describing the deviation of the true activity with field strength by relating the correction factor or mean activity coefficient, $f_{\mp}$ to ionic strength μ. To understand fully the meaning of the theory it is necessary to follow the complete derivation of each of the various equations. Only in this way can one appreciate the assumptions and approximations that were made. Since this information is available in current textbooks (10) we shall concern ourselves only with certain implications of the theory.

It was found that, in very dilute solutions (<0.0005 M), a very simple relation describes the activity of a salt as it relates to varying ionic strength:

$$-\log f_{\pm} = A_{z_+ z_-} \sqrt{\mu} \qquad \text{(I)}$$

where A is a combined constant equal to 0.51 in water at 25° C; z_+ and z_- represent the valence of the positive and negative ions, respectively.

As the concentration of salt increases an additional correction is necessary; allowance for the size of the ions must be made:

$$-\log f_{\pm} = \frac{A_{z_+ z_-} \sqrt{\mu}}{1 + aB \sqrt{\mu}} \qquad \text{(II)}$$

where a is a function of the average effective diameter of the hydrated ions of the electrolyte and B is a combined constant, 3.3×10^7 for water at 25° C.

As the concentration of salt is further increased (approaching 1 M) interaction between the charged ions and the dipolar solvent, in effect, reduces the amount of free solvent, and the activity of the ions is in-

creased This requires the addition of another term to equation (II) utilizing an empirically derived constant C

$$-\log f_{\pm} = \frac{1}{1 + aB\sqrt{\mu}} \sqrt{\mu} + C \qquad \text{(III)}$$

The relation between the values of $-\log f_{\pm}$ calculated from the three equations is given in Figure I 1 for a hypothetical case Observed deviations in activities at any ionic strength are well described by equation (III) As the ionic strength is decreased the term C_μ becomes negligible and equation (III) simplifies to equation (II) At even lower ionic strengths, the term $1 + aB\sqrt{\mu}$ approximates unity and the equation

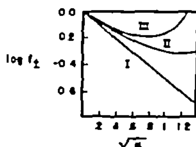


FIG. I 1 —The relation between the values of the mean activity coefficient ($f_{\pm}$) and the square root of the ionic strength as calculated by the simple (*I*) and extended DeBye-Hückel equations (*II*) and (*III*)

simplifies further to equation (I) In very dilute solutions, then, all three equations give equivalent correction factors, as seen in Figure I 1

In practical terms in the range of physiological ionic strength ($\sqrt{\mu} = 0.4$) the effective concentration of an ion in relation to its actual concentration is (*a*) decreased considerably and decreases further with increasing ionic strength and (*b*) decreased even more the greater its valency It is impossible to generalize about effective ion size

DETERMINATION OF ION ACTIVITIES

The mean activity of a simple salt in pure solution can be measured quite precisely by a careful determination of one of the colligative properties of the solution (conductivity for example) The activity of an individual ion, however cannot be measured experimentally Yet it is just this information—the individual ion activities—that we usually require In a complex solution such as serum for example the measure

ment of the mean activity of all the ions present is of almost no value at all. In such a case it is necessary to calculate and interpolate individual ion activities from experimentally determined mean activities of simple salt solutions. To do this, certain conventions must be adopted, and certain simplifying assumptions must be made.

First, the observed activity, a is always expressed as a dimensionless ratio of the true or absolute activity, a_{abs}, in relation to some arbitrary standard or reference state, a_0

$$a = \frac{a_{abs}}{a_0}$$

where a_0 by convention is usually considered to be the activity of the ion in a hypothetical solution of unit (molal) concentration but having the properties of an infinitely dilute solution. Second to convert ion concentration, c to ion activity the *activity coefficient* γ (or f) is employed. This is defined as follows

$$\gamma \cdot c = a \quad \text{or} \quad \gamma = \frac{a}{c}$$

One of the most widely used devices in estimating individual ion activity coefficients makes use of the McInnes assumptions (29)

$$a_{KCl} = a_K = a_{Cl} \qquad \text{(IV)}$$

which states that in a solution of KCl the activities of potassium and chloride are essentially equal (they do show approximately equal ionic mobilities) and therefore, equal to the mean activity of the KCl solution under study

$$\gamma_K \text{ (KCl solution)} = \gamma_K \text{ (other salt solution)} \qquad \text{(V)}$$

which states that the activity coefficient of potassium is the same at any given ionic strength irrespective of the nature of the salts present.

With these assumptions, the activity coefficient of K^+ can be deduced from studies of KCl solutions. This known the activity coefficient of $HPO_4^=$ can be estimated from the mean activity of a solution of KH_2PO_4.

Another method of evaluating individual ion activity coefficients involves calculations employing the Debye-Huckel formulations. Kielland has carried out these calculations for a wide variety of ions (22) Levinskas (26) has performed the tedious task of calculating the activity coefficients of calcium and phosphate ions at physiological ionic

strength from compilations of mean activity data given by Harned and Owen (15)

A collection of the individual ion activity coefficients of a variety of ions of special interest in biological research is assembled in Table I 1 They are useful please note only at physiological ionic strength Note, too the wide variations among ions in the deviation of their activities from their concentrations The activity of the hydrogen ion a_H is 80 per cent of its concentration, $PO_4^{\equiv}$ only 6 per cent.

TABLE I 1

INDIVIDUAL ION ACTIVITY COEFFICIENTS AT PHYSIOLOGICAL IONIC STRENGTH ($\mu = 0.16$)

0.81	H^+	0.33	Sr^{++} Ba Ra
.4	Na HCO_3^-	.32	$CO_3^=$
.72	OH F^- H_2 citrate$^-$ K Cl NO_3^-	.30	H citrate$^=$
.1	Rb Cs NH_4	.36	Ca
.62	$H_2PO_4^-$	.23	$HPO_4^=$
0.40	Mg^{++} Be	.05	Citrate$^{\equiv}$
		0.06	$PO_4^{\equiv}$

Taken from Levinskas (26) all others are graphically interpolated values from Kielland (22)

IMPORTANCE OF ACTIVITY CORRECTIONS

Activity coefficients are of the utmost importance. Without them it would be impossible to generalize data obtained in the research laboratory constants would not be constant and solution chemistry would be a sea of confusing and conflicting data Since these activity corrections are of considerable magnitude they *cannot be ignored* One pertinent example suffices to prove this statement. Consider the solubility product of secondary calcium phosphate $CaHPO_4 \cdot 2H_2O$ The true thermodynamic solubility product $a_{Ca} \times a_{HPO_4^=}$ is constant over a wide range of variation of pH ionic strength and calcium phosphorus solution ratios. If activity corrections are ignored, a measurement of the apparent solubility of this salt at physiological ionic strength would be variable and in error by over 1,200 per cent!

It is difficult enough to detect mistakes. To *commit* errors by the omission of known and established correction factors is inexcusable There are of course many instances in which the necessary corrections and activity coefficients are not yet known In such cases the investigator should, if at all possible maintain the ionic strength con-

stant throughout all experiments. If the experiments are of physiological interest, $\mu = 0.16$ is to be preferred.

Specific Ion Effects

Activity coefficients correct only for *non specific* interactions between ions. In the practical case it is difficult to conceive of a solution containing more than a few simple ions in which no *specific* interaction occurred. When the laboratory experiment is designed to simulate physiological conditions, specific effects are especially important. As an example, the magnitude of the correction factors of both types for calcium in serum may be cited. As shown later only 20 per cent of the total concentration of 10 mg per cent in serum is free, ionic, and effective. A reduction of 36 per cent is the result of non-specific salt effects, interionic attraction a reduction of 44 per cent results from specific interaction, principally binding to serum protein.

At the present time, instances of specific ion effects are so common and familiar that the term *complex ion* is in every scientist's vocabulary Indeed, as college Freshmen we are exposed to the laboratory experiment involving the dissolution of silver chloride precipitates with ammonia by formation of the silver-ammonia complex.

A special kind of complex formation is *chelation*, another familiar term derived from the Greek *chelas* meaning "claw" All effective chelating agents are molecules containing several functional polar groups in such a steric arrangement that they can surround a central ion, thus the term "claw"

Some Important Formation Constants

All such specific ion effects can be considered as examples of the formation of poorly dissociated compounds and can be expressed by the law of mass action in a manner analogous to the formulation for the dissociation of a weak acid. For the general case in which a cation M and an anion, A combine to form a highly associated compound, M_xA_y, where x and y represent the appropriate subscripts, we may write

$$M_xA_y \rightleftharpoons xM^{n+} + yA^{m-} \qquad \text{(VI)}$$

n and m expressing the valence. At equilibrium, the law of mass action can be expressed in activities thus



$$a_{M_xA_y} = K \cdot a^x_{M^{n+}} \cdot a^y_{A^{m-}} \qquad \text{(VII)}$$

where K is the equilibrium constant. If we convert activities to the corresponding concentration-activity coefficient products and then

rearrange we get

$$K = \frac{[M^{+}A_x]}{[M^{+}][A^{-}]^x} \cdot \frac{\gamma_M A_x}{\gamma_M^+ \cdot \gamma_{A^{-}}^x} \qquad \text{(VIII)}$$

where brackets indicate concentrations Because the equilibrium constant is based on thermodynamic considerations of ion activities this is the true association constant K

For the simple case of a non polymeric 1 1 complex—calcium citrate for example—general equation (VIII) simplifies to

$$K = \frac{[\text{Ca citrate}]^{-}}{[\text{Ca}^{++}][\text{citrate}^{\equiv}]} \cdot \frac{\gamma_{\text{Ca citrate}}}{\gamma_{\text{Ca}} \cdot \gamma_{\text{citrate}}} \qquad \text{(IX)}$$

Unfortunately the second term of the right hand side of this equation the ratio of activity coefficients can rarely be evaluated at the present time. This is because the activity coefficient of the complex ion is unknown and cannot even be estimated in most instances. Therefore most investigators who have determined the association of complex ions use a simple K an uncorrected concentration constant or K_f an uncorrected formation constant, to express their results. Here

$$K_f = K \cdot K \quad \text{(ratio of activity coefficients)}$$

The investigator must therefore be on guard in using K_f- or K_f-values. They are valid only when used under the conditions of pH ionic strength and temperature specified If these conditions have not been specified the K or K_f is valueless.

In Table I 2 are assembled published formation constants for a large number of organic chelators of calcium. A perusal of this table shows clearly that the citrate ion is the most potent calcium-complexing agent of all the organic acids yet studied The structure of the calcium citrate complex is not known but a most likely structure can be derived by analogy Detailed studies (7) of the complexing of the uranyl ion by citrate demonstrated a tridentate bonding involving two ionized carboxyl groups and the unshared electrons of the hydroxyl group More recently this same structure has been proposed for the cupric-citrate complex (23) In all probability the calcium citrate complex is similar An examination of the formation constants in Table I 2 shows that those acids which contain two or three carboxyl groups and a hydroxyl sterically available for bonding form the more stable complex ions. For these reasons and from ion-exchange studies which indicate the calcium citrate complex to be of the non polymerized 1 1

type (8 40) and anionic (13), the most likely structure is that given in Figure I 2.

From the formation constants given in Table I 2, another kind of practical information can be deduced. In many instances the investigator requires a buffer with which to maintain the pH control Usually, phosphate buffers are inappropriate in studies of the mineral of bones or teeth. How does one control the pH without becoming involved in specific ion effects? The answer cannot be given categorically of course, because three variables are involved (*a*) the calcium concentration, (*b*) the buffer concentration, and (*c*) the K_f of the calcium buffer complex. In our laboratory 0 001 M diethylbarbiturate in the neutral range and 0 001 M acetic acid in the acid range are suitable buffers,

TABLE I 2

FORMATION CONSTANTS OF ORGANIC CHELATES OF CALCIUM (40)

Citrate	3 15	Itaconate.	1 2
Isocitrate	2 47	Maleate	1 1
Malate	2 06	Succinate	1
Tricarballylate	1 82	Carbonate	0 82
Tartrate	1 78	Lactate	(0 8)
Oxalacetate	1 6	Diethylbarbiturate	(0 66)
α-Carboxypimelate	1 59	Acetate	(0 62)
Aconitate	1 50	Glutarate	0 55
Oxalosuccinate	(1 5)	Fumarate	(0 48)
Malonate	1 36	Aspartate	0 44
Citraconate	1 30	Ascorbate	(0 19)
α-Ketoglutarate	1 29	Glycine	(0)
Gluconate	1 22		

At pH 7.2–7.3, μ = 0 16 and t = 25° C expressed as log K_f Values in parentheses are only approximate

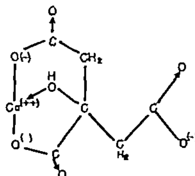


FIG I 2.—A suggested structure of the calcium citrate complex

giving a negligible correction (1 per cent or less) for complex ion formation in solutions containing calcium up to 0.001 M.

In Table I 3 are collected the formation constants of a number of phosphorus-containing chelators of calcium. The most significant conclusion to be drawn here is that calcium is indeed "phosphatophilic." Whereas organic acids require a very specific structure for complex formation with calcium, it appears that almost any phosphate compound has a strong attraction for this cation. Even unadorned ortho-

TABLE I 3

FORMATION CONSTANTS OF PHOSPHORUS-CONTAINING CHELATES OF CALCIUM

Compound	Log K_f	Method	Conditions	Reference
Orthophosphate	1.79			
Trimetaphosphate	2.02			
Tetrametaphosphate	2.[illegible]		$\mu = 0.15$	
Pyrophosphate	3.17	Exchange	$t = 37°$ C	11
Phytate	3.16		pH = 7.4	
Tripolyphosphate	3.64			
Hexametaphosphate	7.62			
Adenosinediphosphate	3.41		$\mu = 0.1$	
Adenosinetriphosphate	3.58	Exchange	$t = 37°$ C	5
			pH = 7.4	
Ribose nucleic acid	1.92	Dialysis	$\mu = 0.15$	
Desoxyribose nucleic acid	1.80	Dialysis	$t = 25°$ C	49
Desoxyribose nucleic acid	1.91	Exchange	pH = 7.2	

phosphate is a fair complexing agent. Polymeric phosphates match or exceed citrate in their ability to complex calcium. The significance, both experimentally and physiologically, of such pronounced specific ion effects is so obvious that it need not be emphasized further.

CALCIUM-PROTEIN INTERACTION

In 1871 Pribram made the first suggestion that part of the calcium in serum might be bound to protein (37). Forty years passed before Rona and Takahashi brought forth the first experimental evidence (38) in support of Pribram's idea. Another twenty-five years passed before the calcium-protein interaction was placed on anything like a quantitative basis. In a monumental classic (33) McLean and Hastings summarized existing data and, by means of the frog heart method (32) for measuring calcium ion activities, assembled tables of dissociation constants based on the empirical mass law formulation.

$$\frac{[Ca^{++}]\ [protein^{-}]}{[Ca\ protein]} = K \qquad (X)$$

Only recently have methods and techniques become sufficiently refined to improve on this simplified formulation. There are two principal reasons why the McLean Hastings formulation has stood the test of time. In the first place, it *does* describe quite accurately the protein binding of calcium in most normal sera and in several disease states. In the second place progress has been severely hampered by the lack of suitable methods for the direct determination of calcium ion activity. Certainly the frog heart procedure has not become popular despite its historical significance and applicability under specified conditions.

Space does not permit a detailed analysis of the reasons why the determination of a_{Ca} has proved so difficult. We can say however, without fear of contradiction that whoever develops a simple, accurate method for the estimation of calcium ion activities will truly be a hero to clinician and researcher alike. Among the more promising prospects in this connection are Carr's collodion membrane electrode (3) so far applicable only to pure solutions, and the possible development of a highly specific dye which complexes calcium in the pH range 7–8.

Returning to the problem of calcium-protein interaction let us examine Tables I 2 and I 3 to find the groups and structures of proteins which might have a strong affinity for calcium. It is clear that calcium shows little ability to chelate with nitrogen or amino compounds (25) illustrated by the K_f of 0 0 recorded for calcium glycinate. Ordinary carboxyl groups are ineffective (acetate) polycarboxylic compounds are more effective (succinate tricarballylate) but most effective are polycarboxylic hydroxylated compounds (maleate, isocitrate, citrate). From this one would expect that only a few of the total number of protein-carboxyl groups would bind calcium strongly—only those which are in close proximity to other carboxyl or hydroxyl groups. Proteins would be expected to vary in their affinity for calcium depending on their amino acid composition and structure. Phosphorylated proteins might have a special affinity. Certainly binding would be expected to be pH-dependent.

These expectations are consistent with experimental results. In Table I-4 are given Carr's (3) maximum binding values for a number of proteins. The values vary from protein to protein, and although structural details have not been related to binding ability proteins do not chelate calcium simply in proportion to their free carboxyls.

Other examples of differences in protein affinity are illustrated in Figures 1-3 and 1-4. Figure 1-4 is of special interest. This shows that at normal serum levels of the calcium ion albumin binds weight for weight nearly twice as much calcium as do the globulins and nearly four times as much as the γ-globulin fraction. One must conclude then, that roughly three fourths of the protein bound calcium is carried by serum albumin *under normal circumstances*.

The importance of pH in calcium binding has been adequately studied only with the albumin-calcium reaction. In this instance binding is insignificant below the isoelectric point, increasing almost linearly

TABLE 1-4

COMPARISON OF MAXIMUM BINDING OF CALCIUM AND MAGNESIUM TO VARIOUS PROTEINS AT pH 7.4

Protein	Moles Ca Bound/10^5 Gm	Moles Mg Bound/10^5 Gm	Max. Binding Capacity (Moles/10^5 Gm) (46)
Serum albumin	10.5	11.3	135
Serum globulins	5.6	6.2	
Hemoglobin	0.0	0.0	113
Egg albumin	5.0	6.4	82
Lysozyme	7.0	7.6	
Pepsin	31.0	34.0	

Derived from Carr (3)

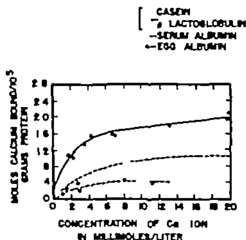


FIG. 1-3.—The binding of calcium to four proteins with similar isoelectric points, pH = 7.4 (3).

with increasing pH (3) up to about pH 8 (21) Since nearly all carboxyls are ionized above pH 5 Katz and Klotz have attributed the binding above pH 5 to increased electrostatic attraction as other acidic groups ionize (21) but this explanation explains' too much. They have resorted to the phenomenon of pH induced swelling of the protein molecule to leaven the electrostatic effect. Thus, in their words, "the decreased affinity for cationic calcium could be attributed to a separation of hydroxylic and carboxylate residues of albumin, associated with the swelling process. *Such a separation would reduce the intrinsic affinity* of each site for the cation and thus counterbalance the increased electrostatic attraction due to the rise in pH" (21)

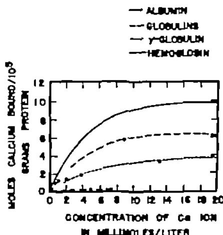


FIG 1-4.—The binding of calcium to various bovine blood proteins. pH = 7 4 (3)

The effect of temperature on calcium-binding by protein has not been studied fully There are indications that temperature is not an important variable for calcium-albumin binding over the range 0°–25° C (21) However significant changes in calcium-binding by serum proteins have been shown over the range 10°–37° C (45)

It should be all too clear at this point that calcium-protein interaction is discouragingly complex. At the risk of adding salt to the already ulcerated condition we must consider two possible technical difficulties which cannot be evaluated at the present time

Martin and Perkins (30) have shown that the same protein will vary in its affinity for calcium, depending on its method of preparation. This is a most obnoxious experimental result. One can obtain interpretable results only with simplified systems (that is physical chemis-

try!) and with purified components (that's common sense!) But if one cannot generalize from the experiment what earthly good can come of it all?

Feldman and Havill (6) have shown that traces of contaminating citrate accompanied protein through a remarkably thorough series of purifications. More important this contaminating citrate was sufficient in quantity to affect studies of metallic ion–protein interaction at least in model systems in vitro It seems possible that the differences observed by Martin and Perkins were actually differences in the contaminants present after different isolation procedures. Contamination may also be related to their finding that albumin shows different binding properties when isolated from abnormal sera (31) Through all these studies there exists the possibility that partial denaturation of the proteins, unavoidable and undetected in most instances may markedly influence results.

However we should not dismiss the findings of Martin and Perkins as merely a problem of contaminants or denaturation. More data are badly needed. It is necessary only to refer to Figure I 3 to remind us that a phosphoprotein—casein—shows a very strong affinity for calcium. The addition to a protein of a few judiciously placed phosphate groups might markedly alter its calcium-binding ability without significantly affecting other properties. An example of such specific structural effects is the vitellin-like protein found in the serum of estrogen treated chickens. This phosphate-rich protein binds fantastic quantities of calcium (39)

In conclusion at the present time we can be certain only that the calcium protein interaction is important and varies with the protein structure. We are not sure how reliable even the most recent data actually are. We can be sure on theoretical grounds, that the McLean Hastings formulations are not an adequate description of the equilibriums involved (21) However the problem of contaminants casts doubt on the present applicability of the more refined formulations given by Klotz (24)

STATE OF CALCIUM IN SERUM

From our discussion of calcium protein interactions it is not surprising that only a rough description can be given of the state of calcium in serum. Part of our difficulty results from our imperfect understanding of the calcium-protein interaction but part of the trouble can be traced to the lack of generally acceptable analytical methodology Every labo-

ratory has its own special modification of one of the three or four basic methods of calcium analysis and this modified procedure is adhered to as rigidly as though it were a religious rite. This practice improves the reproducibility of data from any given laboratory but it does not insure accuracy "Normal" values for total serum calcium vary then, from laboratory to laboratory hovering around a mean of about 10 mg per cent, which is such a nice even number that, correct or not, it is what everyone remembers as the representative value for human serum

Accepting as we must, this value of 10 mg per cent, or 2.5 mM/l, for the total calcium, we are faced with the problem of determining the normal calcium ion activity $a_{Ca^{++}}$ We know the ionic strength of serum and the activity coefficient of Ca^{++} at physiological ionic strength It is easy therefore, to correct for non-specific interionic at traction. How does one correct for specific ion effects, particularly protein-binding?

Lacking a suitable method for the direct evaluation of $a_{Ca^{++}}$ or $[Ca^{++}]$ a number of investigators have resorted to a practical solution by separating the protein-calcium from the diffusible calcium by means of dialysis or ultrafiltration techniques. Of the two ultrafiltration is the more convenient method, particularly with the simplified apparatus described recently (43) Perhaps ultrafiltration would be more widely used if researchers could be reassured of its accuracy and validity Since it is a dynamic process and concentrates the proteins, one might justifiably wonder how well ultrafiltrate values reflect the true state of affairs. If the $[Ca^{++}]$ in the ultrafiltrate were not exactly equal to the $[Ca^{++}]$ in the original serum then the $[Ca^{++}]$ in the remaining unfiltered serum would change during the course of the ultrafiltration In such a case the ratio of free protein to calcium protein would necessarily shift, since

$$K = [Ca^{++}] \frac{[Pr^-]}{[CaPr]} \qquad (XI)$$

Since the $[Ca^{++}]$ in the ultrafiltrate has been found (17 44) to be *independent* of the rate of filtration or the proportion of serum expressed (at least up to two-thirds the total volume) *it follows that* μ K *and* $[Pr^-]/[CaPr]$ all remain constant and that $[Ca^{++}]_{serum} = [Ca^{++}]_{ultrafiltrate}$ throughout the course of ultrafiltration.

Unfortunately much of the data in the literature was obtained with apparatus in which the pH or the temperature was difficult to control.

Since both have been shown to be important variables affecting both protein binding (3) and chelation by serum anions (45) only two recent results can be considered reliable (17 45)[1] calcium in normal sera averages 65 per cent ultrafilterable or 6.5 mg per cent (1 62 mM/l)

Of this diffusible calcium a small fraction is in the form of soluble complex ions, but it is possible only to guess at their concentrations. If one assumes that the anions of serum are complexing only with calcium (ignoring the small error introduced by neglecting the competition of other polyvalent cations magnesium and trace metals) one can calculate the maximum quantity of calcium bound as soluble chelates. Only three of the known anions[2] of serum bind significant quantities of calcium—bicarbonate phosphate and citrate

The state of calcium in normal serum can thus be approximately described as graphically illustrated in Figure I 5 The ionized calcium [Ca^{++}], is estimated by subtracting all known specific ion interactions leaving a total of 1.33 mM/l of ionized calcium This calculated distribution of calcium in serum is in remarkably good agreement with the results published in 1935 by McLean and Hastings using the frog heart method for calcium ion and with the recent studies of Yendt *et al* (50) using as a bioassay technique the mineralization of slices of cartilage from rachitic rats. A comparison of calculated and observed values is given in Table I 5 The amount of ionized calcium in normal serum now seems to be pretty well established—approximately 1.3 mM/l Throughout the subsequent discussion this will be taken as the true *concentration* of free calcium ion Calcium ion activity $a_{Ca^{++}}$ can then be estimated by multiplying the concentration of free ionized calcium by its activity coefficient

$$a_{Ca} = \gamma \quad c = (0\ 36) \times (0\ 0013) = 4\ 7 \times 10^{-}$$

It is not possible to generalize concerning the distribution of calcium in sera of diseased or abnormal metabolic states. Many variables are operative pH A/G ratio [P_{total}] [HCO_3] [citrate$^{=}$] and the pos

1 Howard *et al* found 70 per cent ultrafiltrable *at room temperature* Toribara *et al* found 70 per cent ultrafiltrable *at room temperature* and 65 per cent at 37° C.

2 The following anions were considered (concentrations in serum in mM/l are given in parentheses) phosphate (1) bicarbonate (25) citrate (0.1) succinate (0 04) malate (0 03) fumarate (0.02) alanine (0.004) glutamate (0.002) glycine (0 002) tyrosine (0.001) β-hydroxybutyrate (0.0005) and lactate (0 01) Data taken from Krebs (17) We have neglected the complexes [$Ca_2HPO\ CO_3$] and [$Ca_4PO\ CO^{-}$] proposed by Greenwald (14) because they were based on an assumed K_{sp} for "tricalcium phosphate"—an assumption not valid, as discussed in chap. II

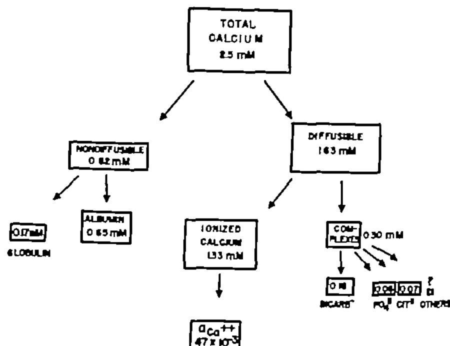


Fig. I 5 —The state of calcium in normal serum as calculated from ultrafiltration data and formation constants. *In ignoring possible ion competition—with* Mg^{++} for example—the amount of calcium estimated to be complexed may be in error (too high) This error could hardly affect the estimated ionized calcium by more than 2 or 3 per cent, however *mM* designates mM/l.

TABLE I-5

Comparison of New and Old Estimations of Distribution of Serum Calcium

Calcium Fraction	Present Calculations (mM)*	McLean-Hastings (12) (mM)†
Total	2 50	2 90
Protein-bound	0 82	1 27
To globulin	0 17	0 47
To albumin	0 65	0 81
Soluble complexes	0 30	0 34‡
Ionic	1 33 | 1 25–1 4§	1 29

* Obtained at 37° C. expressed as mM/liter

† Obtained at room temperature expressed as mM/kg H_2O

‡ Fraction unaccounted for

§ Based on bioassays of Yendt *et al.* (38).

sible presence of abnormal protein It is not a hopeless problem (18), but more study is required

STATE OF PHOSPHATE IN SERUM

The distribution of inorganic phosphate in serum is much less complicated than that of calcium Protein binding is negligible and except for a small fraction complexed by divalent cations (≪10 per cent) as discussed earlier all the inorganic phosphate is ionized.

Since 1920 (4) there have appeared many papers showing that the inorganic phosphate of normal serum is completely ultrafilterable (1 2 17 19 34 36 41 42 46 48) There is some uncertainty regarding corrections for Donnan effects and the volume occupied by the protein but these corrections are of the same order of magnitude as the analytical error A non-filterable complex of colloidal calcium phosphate is well known and occurs in serum with *markedly* elevated calcium or phosphate levels (17) It can also be produced by the addition of calcium or phosphate to normal serum in vitro (17) However such a non filterable complex has never been described in normal serum.

Despite this impressive body of data a number of reports has appeared recently (9 12, 35 43) which purport to show that in vivo a slowly equilibrating non filterable phosphate complex exists in normal serum (12) All these reports present the same kind of indirect evidence based on greater specific activity found in the urine than in the blood after administration of P^{32}-labeled phosphate Since several laboratories (16 20 28) have been unable to confirm these findings and since there are several explanations for the occurrence of specific activity differences between blood and urine, it is quite unnecessary to postulate the existence of a non-filterable phosphate complex. Three explanations come to mind (*a*) The presence of a small amount of P^{32} labeled contaminant in the injection fluid could easily give aberrant urine-blood specific activity ratios. (*b*) No one knows how much time is required for the passage of a phosphate ion from the glomerulus to the ureter If phosphate binds to the tubular surfaces, the lag time between filtration and excretion could be considerable and could introduce apparent urine-blood specific activity differences. (*c*) Finally if there exists any renal mechanism for the tubular *secretion* of phosphate one would expect specific activity differences. Until more substantial more direct, evidence to the contrary is forthcoming we must conclude that the ultrafilterable phosphorus is almost exclusively inorganic phosphate and that all the inorganic phosphorus in normal serum is ultrafilterable

It is difficult to pick precisely a normal value for the total inorganic phosphate of human serum. Unlike serum calcium, the phosphate concentration varies a great deal with age and metabolic state particularly. Most clinicians would agree that a value of 6.2 mg. per cent is a high normal for the newborn, that 2.5 mg. per cent is a low value for a normal adult, and that 3.5 mg. per cent is a good over-all average fasting value for adults. It is important to agree on this point, because later when solubility is under discussion these values must be critically employed. It should be acceptable to select two values to set the limits of normal variation: 1 mM/l (3.1 mg. per cent) as a lower limit or "adult fasting value" and 2 mM/l (6.2 mg. per cent) as an upper limit or "infant value."

There remains the problem of determining the state of ionization of orthophosphate in serum. Levinskas (26) has derived a convenient method of calculating the concentrations of the various forms of orthophosphate at $\mu = 0.165$ and 37° C. He has employed thermodynamic association constants and activity coefficients in his derivation, leaving pH as the only variable.

$$\log \frac{[HPO_4^{=}]}{[H_2PO_4^{-}]} = pH - 6.76$$

$$\log \frac{[PO_4^{\equiv}]}{[HPO_4^{=}]} = pH - 11.42$$

From these relations it is easy to derive the percentages, concentrations, and thermodynamic activities as assembled in Table I-6. Those who are familiar with buffer ratios given by the traditional Henderson-Hasselbalch equation (at pH 7.4, $HPO_4^{=}/H_2PO_4^{-} \simeq 4$) will perhaps be surprised to learn that, in terms of *activities*, there is little difference between the two ions $[H_2PO_4^{-}]$ and $[HPO_4^{=}]$: 0.12 and 0.19 mM/l, respectively.

Thermodynamic Product of Calcium and Phosphate in Normal Serum

Many of the concepts to be developed in subsequent chapters are based on the degree of saturation of serum and inorganic solutions with respect to calcium and inorganic phosphate. As we have seen, the degrees of saturation of solutions are best compared in thermodynamic terms—in ion activities. In this instance we must select an activity *product*. The question is, What product shall be selected? A solution can be saturated only with respect to a defined solid phase.

As shown later only one solid phase of calcium and phosphate is stable under physiological conditions—hydroxy apatite. Levinskas (26) demonstrated that of all the possible activity products one might select, the simplest, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ showed the least variation in describing the solubility of hydroxy apatite. Moreover it is shown later that the K_{sp} of $CaHPO_4 \cdot 2H_2O$ ($a_{Ca^{++}} \cdot a_{HPO_4^{=}}$) also governs the stability of neutral solutions of calcium and inorganic phosphate when no solid phase is present.

For these reasons the degree of saturation of serum and inorganic solutions will subsequently be expressed in terms of this simple product, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ From the data given in Table I-6 and the calcium activity (0.47×10^{-3}) derived earlier we obtain the values of 0.89×10^{-7} for normal fasting adult serum and 1.7×10^{-7} for normal infant serum.

CHAPTER II

Solubilities

We have devoted considerable space to clarifying the state of calcium and phosphate in normal serum, arriving at a thermodynamic expression of the degree of saturation as $a_{Ca} \cdot a_{HPO_4} = 0.917 \times 10^{-7}$. Now we must turn to the solubility of the mineral phase of bone itself. A clear concept of the solubility of bone mineral is prerequisite to an understanding of the biochemistry of calcium and phosphate and the physiology of bone. Consider the matter of calcification: does it not involve solubilities? Consider the maintenance of the serum levels of calcium and phosphate from the mineral reservoirs of the skeleton. Consider the absorption of calcium and phosphate from the intestinal lumen. Consider the regulatory actions of the parathyroid hormone and vitamin D on these processes. Do not all these problems require an understanding of the solubility relationships governing the dissolution and formation of the calcium phosphate minerals?

And what is our understanding of these solubility relationships? Is there a generally accepted comprehensive concept which enjoys the support of a body of well-conceived and well-executed experimental evidence? The answer is, of course, a most emphatic No! The literature on this complicated subject is so confusing and conflicting that most workers in this field have adopted an ostrich like attitude, ignoring the problem of solubility in the self-deluded hope that "maybe it will go away." Most certainly the problem will not go away, and we must do the best we can to evolve a rational picture which fits those data presently available. Let us begin at the beginning with a brief perusal of the general laws of solubility.

SOLUBILITY THEORY

SOLUBILITY PRODUCT CONSTANT[1]

The ions of a solid, like those of a liquid, have a certain tendency to escape, to evaporate, because of the thermal agitation of molecules. In

1 This discussion ignores solvation effects. This is permissible only under equilibrium conditions. The change in solvation of the ions in going from crystal to solution is important in determining the heat of solution, temperature coefficient, etc.

a confined system at fixed temperature this escaping tendency is a fixed quantity a constant vapor pressure in the case of a pure liquid in contact with a gas, a fixed solubility in the case of a pure solid in contact with a solvent. The vapor pressure is independent of the amount of pure liquid present because, though the number of molecules escaping is proportional to the interfacial surface area, so also is the number of molecules returning by collision. Increasing the surface area of the liquid present increases the speed at which equilibrium is reached, but it does not affect the vapor pressure attained at equilibrium. Parallel rules apply to the solubility of a pure solid.

These considerations form the basis for the development of the concept of the *solubility product constant* Consider the dissolution of a simple salt of defined crystal form MA At equilibrium the dissolution may be expressed by the equation



$$\underset{\text{(solid)}}{MA} \rightleftharpoons \underset{\text{(solution)}}{[MA]} \qquad \text{(I)}$$

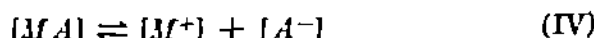
or

$$K = \frac{a_{MA\ (\text{solution})}}{a_{MA\ (\text{solid})}} \qquad \text{(II)}$$

where a is the activity of the salt in solution and the equivalent activity or escaping tendency of the solid. Since the escaping tendency of the pure solid is constant with constant temperature the activity of the solid is constant and is, by convention, assigned a value of unity which simplifies equation (II) to

$$K = a_{MA} \qquad \text{(III)}$$

Simple salts dissociate in aqueous solution and, at equilibrium this may be represented by



$$[MA] \rightleftharpoons [M^+] + [A^-] \qquad \text{(IV)}$$

or

$$K_d = \frac{a_{M^+} \cdot a_{A^-}}{a_{MA}} \qquad \text{(V)}$$

Combining equations (III) and (V) we obtain

$$K_d \cdot K = a_{M^+} \cdot a_{A^-} = K_{sp} \qquad \text{(VI)}$$

which states that, at a given temperature the thermodynamic product of the ion activities in any solution in contact with a pure binary solid is always a constant at equilibrium.

From this derivation it is easily seen that the principle of a solubility

product constant K_{sp} is applicable and valid only if the activity of the solid phase remains constant

ABERRANT SOLUBILITY

There are two factors besides temperature which influence the activity or escaping tendency of a solid Opposing the thermal escape is the interionic attraction of the ions in the crystalline lattice If the crystal is extremely small the surface ions are not restrained by attraction to underlying ions to the extent that they are in an infinitely large crystal Small particles therefore show an aberrantly high surface energy escaping tendency and solubility Furthermore if any foreign ions are present in the lattice the restraining force is altered and solubility is either increased or decreased depending on their effect on the interionic attraction in the crystal

In the case of substitution by ions foreign to the lattice, there are so many possible effects both in kind and in degree that no one has attempted to derive a quantitative formulation in mathematical terms. The effect of particle size, however has been studied and a number of formulations have been made beginning with Ostwald (12) Freundlich (2) Jones (6) and Gross (4) have presented modified equations, but all these derivations suffer from a major shortcoming Based on surface energy they predict decreasing solubility as the particle size increases to its "infinite dimensions (an observable fact) conversely they predict infinite solubility at "infinitely small particle diameters, which is not consistent with the observation that extremely small particles frequently exhibit a very low solubility The radiocolloids, for example, dissolve to a fantastically small extent (14) The hydroxy apatite of bone also occurs as colloidal crystals of low solubility This paradox has been resolved by Knapp who considered in addition to surface energy the effects of electric charge on the solubility of particles of varying dimensions. He obtained an equation in exponential form (7)

$$S = S \; e^{(\alpha/\;) - (\beta/r^4)}$$

where S is the observed solubility and S is the normal solubility or K_{sp} of macrocrystals. In the exponent term $\alpha = 2\,\sigma M/RT\rho$ and $\beta = q^2 M/8KRT\rho$ where ρ is the density σ is the interfacial surface tension, solid/liquid q is the electric charge on a particle of radius r K is the dielectric constant of the substance R is the gas constant T is the absolute temperature and M is the molecular weight of the salt in solution

Knapp's equation has not received the attention it deserves, for it is of the greatest fundamental significance if correct. When published in 1922 the equations could not be conveniently tested experimentally. Crucial experiments required particle-size characterizations in the colloidal or subcolloidal range, and methods for such characterizations are only now being evolved. Besides, methods of separating the solution from the colloidal solid phase and of analyzing extremely dilute solutions were crude or non-existent. Today with new filtration and centrifugation techniques and with carrier free isotopes readily available, the mathematical derivations could be rigorously examined in the laboratory. Unfortunately because the ideas were presented before their time they lie, dust-covered and forgotten, on the back shelves of libraries all over the world.

We have no way of knowing at present, then whether Knapp's development is correct or not. However it does provide a convenient and systematic description of the solubility of extremely small particles. While differing from previous formulations only in considering electric charge it seems to fit observed results much better. We shall use it then, with due reservations.

CHARGE, PARTICLE SIZE AND SEEDING

To understand fully the meaning of Knapp's general equation it is important to keep in mind that it describes only the final solubility value found in a solution in equilibrium with a solid phase of defined particle size and composition. It tells us nothing of the relative proportions of material in the two phases. It might be a barrel of solution in equilibrium with a single crystal or a barrel of crystals in equilibrium with a single drop of solution. Knapp did not define accurately what he meant by S or S_r, but by inference, S could be nothing other than the activity product $a_{M^+} \cdot a_{A^-}$ defined by the K_{sp} of salt MA. For our special interest we could substitute, then $a_{Ca^{++}} \cdot a_{HPO_4^{--}}$ for S_r. The general equation, in theory describes the equilibrium attained either by the dissolution of solid particles or by the precipitation of solid from solution. Conceptually it is simpler to understand the course of events if we think of the equilibrium as being approached by *the formation of* a small number of solid particles in a large volume of solution experimentally prepared. The predicted course of events can then be deduced by reference to a graphic representation of Knapp's general equation as given in Figure II 1.

Starting near the origin point A it is seen that, even if we mix ex

tremely dilute solutions of the salt in question some degree of aggregation of ions is possible. Solid particles to be stable, must be extremely minute of the order of dimers and trimers. As we increase the concentration of ions in solution from point B to C the solution may be considered metastable the actual value of S exceeds the K_{sp}—i.e S Yet the solid phase can be present provided that the particles have a radius lying between r_1 and r_2 These particles will not grow spontaneously because the larger sizes are more soluble than the smaller sizes. In fact at a given solution product S the particles will tend to become uniform in size because small sizes will grow at the expense of larger ones.

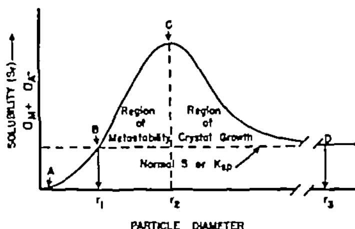


FIG. II 1—A graphical representation of the theoretical relation between particle size and solubility of a given salt as predicted by Knapp's equation (7)

At point C the situation reverses. This is the point of spontaneous precipitation. The equilibrium particle size (r_2) is now sufficiently large that the particle is *approaching the properties of an infinitely large* crystal. The larger particles are less soluble than the smaller ones and will grow at their expense Given time then the mixture will by itself follow the curve from point C to D forming macrocrystals and finally exhibiting a normal solubility or K_{sp}. The curve *between* C and D is largely hypothetical since an equilibrium condition could occur only in the unlikely event that crystals (between r_2 and r_3) *all of the same size* were added to a solution containing the appropriate ion product.

In the metastable range between B and C it is easily seen that, if one introduces macrocrystals or materials isomorphic to the substance under study or in any way increases the state of aggregation of the particles (greater than r_2) the condition of metastability is destroyed.

Crystallization then occurs at ion products below the point required for spontaneous precipitation (point *C*) because of the shift from region *BC* to region *CD* Such an induction of crystal growth in metastable solutions is termed ' seeding

Note, however that it is possible for colloidal particles of one particle size, r_1 to coexist with macroparticles of size r_2 even at equilibrium.

SOLUBILITY OF CALCIUM PHOSPHATE–AQUEOUS SYSTEM

With this brief review of solubility theory let us turn to the specific problem of evaluating the solubility of the calcium phosphate-aqueous system. In vivo this system appears to be quite unruly The clinician can cite instances in which the levels of both calcium and phosphate ions in serum are abnormally elevated or abnormally depressed. In some cases the serum levels change reciprocally In vitro the system is also unruly Investigators, studying the dissolution of bone mineral obtain variable and unpredictable results (10) Even if the problem is simplified to the point of using preparations of pure synthetic hydroxy apatite in water the dissolution data cannot be systematized (8)

The solubility of the calcium phosphate system is confusing only at near neutral or alkaline pH's. At very low pH (<4.0) the system is governed by a normal K_{sp}—that of $Ca(H_2PO_4)_2 \cdot H_2O$ primary calcium phosphate At a higher pH (up to 6 0) the system is governed by the K_{sp} of $CaHPO_4 \cdot 2H_2O$ secondary calcium phosphate. Above 6.2, however secondary calcium phosphate is unstable and undergoes spontaneous hydrolysis. The hydrolysis product always exhibits the crystal structure of hydroxy apatite $Ca_{10}(PO_4)_6(OH)_2$ a compound which is variable in composition and forms only extremely small particles Thus, as we approach equilibrium starting with a mixture of solutions, we are dealing with the K_{sp}, $a_{Ca^{++}} \cdot a_{HPO}$ The final equilibrium however is governed by hydroxy apatite, which has no K_{sp} and possesses all the characteristics contributing to aberrant solubility The spontaneous hydrolysis of $CaHPO_4 \cdot 2H_2O$ introduces a discontinuity into the system. The laws governing the system when solid is absent differ from those operating when solid is present. One cannot approach the same *equilibrium from the two directions—precipitation* and dissolution.

THEORETICAL DEVELOPMENT

This combination of factors can be described in terms of Knapp s equation, as in Figure II 2 As one mixes neutral solutions containing increasing amounts of calcium and phosphate, curve *A* the solubility

curve of $CaHPO_4 \cdot 2H_2O$ is followed to the point of spontaneous crystallization point *C* where solid separates. However if the pH is above 6.2 these crystals rapidly hydrolyze to hydroxy apatite. If the pH were below 6.2, the system would follow curve *A* to the normal K_{sp}, with the growth of macrocrystals of $CaHPO_4 \cdot 2H_2O$. The formation of the tiny apatite crystal however places the equilibrium out of control of the Knapp relation for $CaHPO_4 \cdot 2H_2O$. Instead, the equilibrium is governed by some hypothetical curve curve *B* in Figure II 2. The numerous crystal nuclei formed when $CaHPO_4 \cdot 2H_2O$ hydrolyzes result in a sharp drop in the $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ of the solution. This is indicated by the downward arrows, curve *B*.

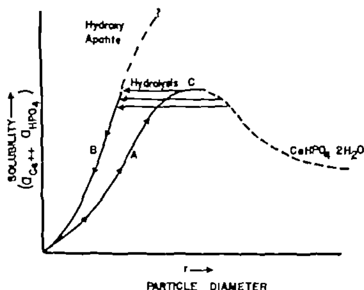


FIG. II 2.—The relation between particle size and solubility (after Knapp) showing the conversion of solid phase from $CaHPO_4 \cdot 2H_2O$ to hydroxy apatite above pH 6.2. Hydroxy apatite crystals formed from solution are of colloidal dimensions and, because of the shift from the right-hand side of curve *A* to the left-hand side of curve *B* are unable to attain macrocrystalline dimensions

One might reasonably inquire why Knapp's equation cannot be applied to the hydroxy apatite system itself by substituting the product $a_{Ca^{++}}^{10} \cdot a_{PO_4^{\equiv}}^{6} \cdot a_{OH^-}^{2}$ for the value S_r. There are two good reasons. First, the smallest unit of structure, $Ca_{10}(PO_4)_6(OH)_2$ would involve in its formation a simultaneous collision of *eighteen ions* all of proper activation energy. This is an outright impossibility on statistical grounds. Second, the crystal lattice itself is capable of wide variations

in chemical composition. For this reason, no single ion product, even if expressed in activities, can be expected to describe the solubility behavior of hydroxy apatite. In actual laboratory experience, Levinskas (8) found that the simplest ion product, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ showed the least variation of all those tested, in describing dissolution equilibriums of hydroxy apatite. Since this same product governs the stability of solutions in the absence of the solid phase we shall continue to use it as an expression of the degree of saturation of inorganic solutions and serum.

No one has ever succeeded in obtaining crystals of hydroxy apatite larger than 0 1 mm. in length even with the aid of high temperatures and pressures (13) Most hydroxy apatite particles are in the range of colloidal or near-colloidal sizes, which as we have seen do not exhibit normal solubility or obey a fixed K_{sp}. Furthermore since the precipitation of secondary calcium phosphate prevents one from preparing any solution beyond the range of metastability with respect to hydroxy apatite, all solutions remain in the range where the *small particles of hydroxy apatite are less soluble than the large particles* This means that the introduction of a seeding material into such a solution causes new crystals to grow even at the expense of larger preformed crystals.

EXPERIMENTAL EVIDENCE

While the preceding development is based on an untested equation, much of what has been described can be supported by experimental findings. Recent studies by Strates (15) have determined the point of spontaneous crystallization when solutions of calcium and phosphate are mixed These data are summarized in Figure II 3 The product $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ required to obtain self-seeding was seen to vary with pH and temperature, but it never fell below 3×10^{-7} All values were greater than the K_{sp} of $CaHPO_4 \cdot 2H_2O$ 2.3×10^{-7} The solid which formed just after mixing exhibited a Ca/P molar ratio of unity At pH 6.2 the solid was stable, and macrocrystals of $CaHPO_4 \cdot 2H_2O$ formed. At pH 6.9 or higher however the solid phase rapidly hydrolyzed to hydroxy apatite with higher Ca/P ratios, 1 4–1 6 This hydrolytic conversion of the initial precipitate to hydroxy apatite was confirmed by an X ray analysis (15) of the solid phase. At equilibrium those solutions which were in contact with crystalline $CaHPO_4 \cdot 2H_2O$ showed a constant value for the product $a_{Ca^{++}} \cdot a_{HPO^{=}}$ which was equal to the K_{sp} of $CaHPO_4 \cdot 2H_2O$ Those solutions in contact with

hydroxy apatite showed widely varying values for this product bu general $a_{Ca^{++}} \cdot a_{HPO_4^=}$ was much less than 1×10^{-7}

Levinskas made a careful study of the dissolution equilibrium well-characterized, essentially "pure" (cf chap iii) hydroxy ap crystals (8) He found that no simple relation could be derived to scribe the observed solubilities. Certain generalizations coulc drawn however and these generalizations confirm the publishec perience of many investigators (10)

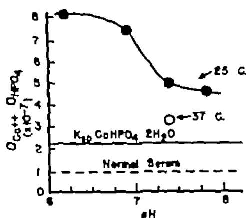


FIG. II 3 —The effect of pH and temperature on the precipitation point. ...at in all instances the precipitation point is greater than the ion product of r ...rum and the K_{sp} $CaHPO_4 \cdot 2H_2O$

...ime To Reach Equilibrium

The dissolution equilibrium between stable preformed crystal ...vater can be attained in a matter of hours (8, 17) On the other l ...hen equilibrium is approached via precipitation from supersatu ...olutions, the equilibrium may not be reached for months (3) Ir ...pitating the mass of tiny imperfect crystals must age, resulti ...hanging surfaces, changing size, and hence changing solubilities ...xtended periods of time.

Effect of Solid-to-Solution Ratio

Knapp's equation predicts that the apparent solubility of col particles will vary with the experimental ratio mass of solid/volu solution. If a small charge of crystals is placed in a large volume lution, the average crystal size (and hence S) will be reduced i process of dissolving On the other hand, with a large charge of cry

apparent solubility of hydroxy apatite $[H^+]^2$ $[Na^+]$ $[Mg^{++}]$ $[CO_3^-]$ and [citrate$^=$] (10) Fluoride ions decrease the effective solubility (16) A number of other ions are known to be able to substitute for the calcium ion in the lattice, but their effect on solubility is not clearly established Pb^{++} Sr^{++} Ra^{++} UO_2^{++} Under physiological conditions, the effect of these ions on the solubility of bone mineral can be ignored.

Solubility under Physiological Conditions

The small particle size of the hydroxy apatite crystals and the host of variables affecting the solubility of hydroxy apatite render it difficult to predict the solubility to be expected of bone mineral under physiological conditions. The very early workers in this field gained the impression that normal serum is supersaturated with respect to bone mineral Recent results (9 11) obtained with some care to control the many variables, confirm this early view

Experimental Evidence

The following evidence may be cited (9) Twenty milligram samples of powdered defatted fresh cortical rabbit bone were suspended in a solution the pH and composition of which corresponded closely to an *ultrafiltrate of normal serum This solution, by itself was stable for* months. After the suspended bone samples had been agitated for 24 hours, they were centrifuged, the supernatant solutions aspirated for analysis, and fresh ultrafiltrate added to the bone powder residues. This process was repeated daily for 17 days. After the first few days steady levels of calcium and phosphate were observed in the ultrafiltrate solution, 1 8 and 4 1 mg per cent, respectively These results are illustrated in chapter vii, Figure VII 3 Consider the variables the pH was 7 4 the ionic strength was 0 16 the temperature was 37° C the Ca/P ratio was near physiological the principal foreign ions (Na^+ CO_3^- Mg^{++} and citrate$^=$) were all present at normal serum levels. Even the solid-solution ratio was rendered unimportant as a variable by starting with supersaturated solutions and reproducing the steady state daily Under these experimental conditions the solubility of bone mineral, expressed as $a_{Ca^{++}}$ a_{HPO} *was* $0\ 49 \times 10^{-7}$ *This product* is half the value calculated for normal serum. Nordin, in an extensive study of the variables affecting bone-salt solubility (11) reached this same conclusion *normal serum is supersaturated with respect to bone mineral*

2. Discussed under effect of pH.

There have been many attempts to relate the serum levels of calcium and phosphate to the solid phase $CaHPO_4 \cdot 2H_2O$ There have also been many unsuccessful attempts to detect the presence of $CaHPO_4 \cdot 2H_2O$ in bone and other calcifications of animal origin Hodge (5) has summarized these data. From the foregoing evidence it is clear that $CaHPO_4 \cdot 2H_2O$ could never be found. In the first place the $a_{Ca} \cdot a_{HPO_4}$ of the body fluids is below the K_{sp} and therefore this salt cannot be formed. In the second place if it should form under abnormal circumstances, it would spontaneously hydrolyze to hydroxy apatite because the physiological pH is more alkaline than 6.2. *Hydroxy apatite is the only solid phase of calcium and phosphate that can form under physiological conditions* (1)

In purely inorganic solutions where foreign ions are absent, pure hydroxy apatite crystals show a very low solubility Usually the equilibrium product $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ is less than one-tenth that of normal serum. On this basis serum is highly supersaturated. Apparently *the presence of carbonate Mg^{++} Na^{+} and citrate greatly increases the effective solubility of bone hydroxy apatite though not sufficiently to account for normal serum levels of calcium and phosphate*

IMPLICATIONS

If we may conclude that serum is supersaturated with respect to bone mineral we may also draw some very important inferences concerning the mechanism of the regulation of bone-blood interrelations. There is ample evidence to believe that for practical purposes, the serum and the extracellular fluid are nearly equivalent in composition. Certainly with respect to pH, Ca^{++} P_{total} Na^{+} etc. the differences between the two fluid compartments are minor If the serum is supersaturated then so also is the extracellular fluid However it is inconceivable that the mineral phase of bone is not in a moment to-moment equilibrium with its own fluid environment. The interstitial fluid of bone cannot be supersaturated with respect to bone mineral unless crystal formation is taking place It follows, therefore, that *the interstitial fluid of bone cannot be equivalent to the extracellular fluid in ionic composition* There must be some diffusion barrier some ion gradient or ion pump between these two fluid compartments. Such a barrier or gradient could be maintained only by cellular activity Such a physiological mechanism provides a rational explanation for the regulatory actions of vitamin D and parathyroid hormone. If serum reflected in a simple and direct way the solubility of bone mineral it is difficult to

understand how a vitamin or a hormone could influence the solubility equilibrium How much more reasonable it is to postulate that vitamin D and parathyroid hormone affect the metabolic activity of those cells responsible for maintaining the ionic gradient between interstitial bone fluid and the extracellular fluid compartment This aspect will be developed more fully in chapter vi. Similarly if serum is supersaturated with respect to the only solid phase stable at physiological pH—hydroxy apatite—some special mechanism must be postulated in the intestine to (*a*) insure a corresponding or greater state of supersaturation in the intestinal contents or (*b*) accomplish a transfer of calcium and phosphate to the portal circulation against an unfavorable ion gradient of calcium and phosphate. This, too is discussed more fully in chapter vi.

Finally the mineralization process itself is placed in new perspective. If the serum is supersaturated, yet below the point of spontaneous precipitation we need not postulate a booster mechanism to elevate locally the levels of calcium and phosphate. They are normally sufficiently high to promote growth of crystals. Attention should be focused instead on the extracellular osteoid matrix. It must possess the ability to seed or induce crystal formation from the saturated or super saturated fluids. This problem is developed more fully in chapter vii. In thinking about the matters to be presented in subsequent chapters—the chemistry of bone mineral the maturation of the skeleton, physiological homeostasis, and the mineralization process—as a single unified concept, the reader may become somewhat confused about solubility interrelations. For example, in this chapter the serum has been shown to be supersaturated. In chapter v the continuing maturation of the mineral of established bone will be attributed to this supersaturation of the body fluids. In considering regulatory mechanisms (chap vi) however the idea will be developed that vitamin D and parathyroid hormone cause the dissolution of bone mineral by producing a condition of undersaturation in the bone fluids. Finally the mineralization process will be described as a catalyzed formation of crystals from super saturated solutions.

This seemingly paradoxical state of affairs can be resolved only by reference to the composition of the various body fluids with respect to ions other than calcium and phosphate. Since bone mineral does not exhibit a fixed K_{sp}, *a given activity product* $a_{Ca^{++}}$ $a_{HPO_4^{=}}$ *cannot be defined as to its degree of saturation unless the concentrations of* Na^+ Mg^{++} $CO_3^{=}$ H^+ *and citrate*$^{\equiv}$ *are also given* The product $a_{Ca^{++}}$ $a_{HPO_4^{=}}$ of normal serum (1×10^{-7}) then, is supersaturated in the serum itself,

supersaturated in areas of forming bone undersaturated in areas of resorption and probably varies in areas of established bone but on the whole tends to be supersaturated This peculiar situation arises from the fact that the concentrations of $CO_3^=$ H^+, and especially citrate$^≡$ differ in these different areas. At the moment we cannot evaluate the contribution of H^+ and $CO_3^=$ to these variations, but there is good reason to believe that the production of citrate by bone cells under the control of the parathyroids and with the assistance of vitamin D is a

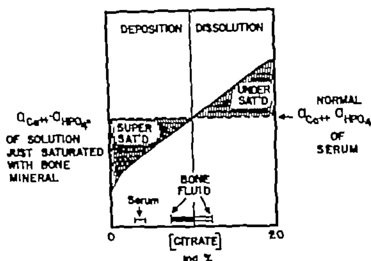


FIG. II 7 —A diagrammatic representation of the relation between the degree of saturation of the product $a_{Ca^{++}} \cdot a_{HPO_4^=}$ and citrate concentration. It has been assumed that the concentrations of Na^+ Mg^{++} $CO_3^=$, and H^+ are constant and equal to normal serum values. Note that the normal product $a_{Ca^{++}} \cdot a_{HPO_4^=}$ may be regarded as undersaturated, just saturated, or supersaturated, depending on the citrate level

most important determinant of the degree of saturation of a given product, $a_{Ca^{++}} \cdot a_{HPO_4^=}$ This relationship between citrate concentration and the relative degree of saturation is illustrated in Figure II 7

The differentials in citrate concentrations from bone area to bone area and from the bone fluid compartment to the extracellular fluid compartment which are largely responsible for the transfer of calcium and phosphate (dissolution and deposition) will be termed 'blood-bone gradients' in succeeding chapters. This non-specific term is meant to remind the reader that non-inorganic ions other than citrate (H^+ $CO_3^=$, possibly lactate) may be playing an important part in determining

whether a given product, $a_{Ca^{++}} \cdot a_{HPO_4^{--}}$ in a given histological location is undersaturated, saturated, or supersaturated.

The actual gradients involved may be quite small, since citrate is so effective in altering apatite solubility. The corresponding gradients in inorganic ions, when shared according to the laws of the Donnan equilibrium, may be very small indeed. From this we may conclude that the product $a_{Ca^{++}} \cdot a_{HPO_4^{--}}$ is essentially the same in all areas: bone fluids, extracellular fluid, and serum.

CHAPTER III

The Mineral Phase

We have seen that hydroxy apatite is the only solid phase of the Ca PO_4-H_2O system which is stable at neutral pH Other more acid salts spontaneously hydrolyze to produce hydroxy apatite. It is appropriate, therefore that we review briefly the crystallography of this material and its relation to the mineral substance of bone The task is not easy as the very name apatite" indicates. Coined by Werner in 1790 (24) the term is derived from the Greek word meaning to deceive." This class of minerals has lived up to its name deceiving and confusing mineralogists, as it has chemists, throughout the years. It is difficult to attain a clear-cut comprehension of the crystallography of hydroxy apatite when the mineralogists themselves do not agree on rather fundamental interpretations. Until agreement is reached, therefore, it is possible only to list the various points of view and show how they differ and how surprisingly much they agree.

The Hydroxy Apatite Lattice

The structural family known as apatites gives an X ray diffraction pattern which except for minor variations, is common to all its members. Characteristically the group occurs as extremely small or poorly developed crystals which are inadequate for precise structural study One member—fluorapatite—does occur naturally as a macrocrystal suitable for accurate analysis. This is the only apatite, therefore for which a detailed structure is available It is reasonably assumed because of the similarity in X ray diffraction patterns, that other apatites possess a structure analogous to fluorapatite In the case of hydroxy apatite, the hydroxyl groups would occupy the positions indicated as fluoride in the following figures.

The structure of the smallest repeating structural unit, or unit cell of fluorapatite, originally proposed by Náray Szabó (31) has been repeatedly confirmed (15, 19 20 23 30) with small alterations proposed for the positions of calcium and phosphorus (3 5) This structure is de-

scribed in Table III 1, and a plane cut across the *c* axis is pictured graphically according to Carlström (8) in Figure III 1 The synthesis of a three-dimensional structure from X ray data is always an involved process, and when the structure is as complicated as that of fluorapatite, it is to be expected that small adjustments in the atomic parameters may be required from time to time as more refined measurements become available.

Because of its complexity it is helpful, along with the detailed dia-

TABLE III 1

PARAMETERS OF ATOMS IN FLUORAPATITE

	x/	y/	/c	
2 F	0	0	0 250	
4 Ca_I	0 333	0 333	0	
6 Ca_{II}	250	0	250	$a = 9\ 37 \pm 0\ 01$†
6 P	416	361	250	
6 O_I	333	500	250	$c = 6\ 88 \pm 0\ 01$†
6 O_{II}	600	467	250	
12 O_{III}	0 333	0 250	0 062	

According to Náray Szabó (31)

† Unit cell dimensions are given in angstrom units, A.

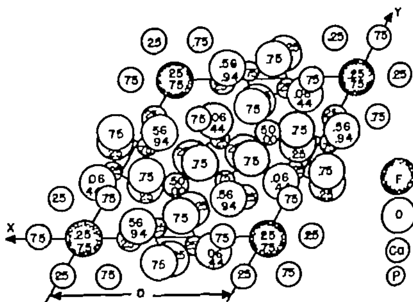


FIG. III 1 —Projection on the (001) plane of the atomic arrangement in fluorapatite. The figures are fractions of the height of the unit cell.

gram to refer to a shorthand representation such as that employed by Arnold (1) This abbreviated unit cell is shown in Figure III 2 It is impossible to explain the three-dimensional arrangement of ions verbally If the reader wishes to gain a full appreciation of this complicated structure, he must consult the original literature and construct actual scale models. The abbreviated representation does differentiate clearly between the triplet or screw axis calcium positions arranged spirally around the fluoride ions and the column calcium positions. The latter may be considered to link together planes of the crystals, which are the parallelepipeds formed by the fluoride ions. The empirical formula for a fluorapatite unit cell then is $Ca_{10}(PO_4)_6F_2$. By analogy hydroxy apatite should have as its unit cell the composition $Ca_{10}(PO_4)_6(OH)_2$

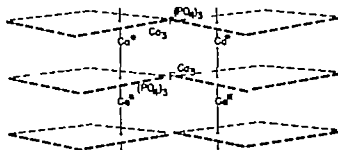


FIG. III 2.—A shorthand representation of the fluorapatite unit cell the *c*-axis running vertically The unlabeled positions are similar to those labeled but should be considered as belonging to adjacent unit cells. *Ca* designates column positions.

LACK OF STOICHIOMETRY

The Ca/P molar ratio of all apatites should be 1 67 and there is no structural evidence for the occurrence of water of crystallization in the lattice. Unfortunately the composition of most apatite preparations does not agree with the composition predicted from the assumed structure. In fact, there exists an array of materials with Ca/P ratios varying from 1.3 to over 2.0 all of which lose significant quantities of water on prolonged heating Yet all these exhibit the X ray diffraction pattern of apatite. It is this divergence between the observed composition and that predicted by structural considerations that provides the basis for the diverse names and structures proposed for bone mineral and the lack of agreement even among mineralogists on the crystallography of the apatite family of minerals.

There are three mechanisms by which the composition might vary without markedly affecting the apparent structure (*a*) substitution of

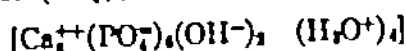
one ion or group for another within the lattice (principally H_3O^+ for Ca^{++}) (*b*) the presence of unsubstituted defects in the internal lattice and (*c*) surface substitution exchange, or adsorption All three explanations seem reasonable and from available evidence likely Yet the crystallographers frequently champion one explanation to the *exclusion* of the other two For example, McConnell and co-authors (25–29) have consistently felt that isomorphic substitution within the lattice is *the* explanation while Carlström (8) shows that surface reactions alone could explain the variable stoichiometry Posner and associates (38) currently propose the term pseudo-apatite as a descriptive name for defective apatites. Finally Hendricks and Hill, who at one time strongly argued for isomorphic substitution as an important property of the apatites (18 19) more recently have favored surface adsorption (17)

We shall assume in this discussion that all these investigators are essentially correct Possibly the differences in emphasis and interpretation may be attributed to differences in the method of preparation or origin of this deceptive substance. Geological specimens which are very old and probably formed at high temperature can be expected to have few internal defects. Fusion of crystals would minimize the importance of surface reactions. Here intracrystalline substitutions would be relatively more important. On the other hand freshly formed precipitates have a great many voids and imperfections, as indicated by their diffuse X ray diffraction pattern. They consist of extremely small particles with an enormous specific surface. Here defects and surface substitutions dominate the aberrant stoichiometry By sifting the published results, it is possible to show that all three mechanisms for variable composition are probably operative.

INTERNAL LATTICE SUBSTITUTION

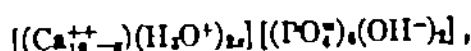
Kunin and co-workers (21) slowly precipitated hydroxy apatite at elevated temperature (100° C) with the pH carefully controlled. Relatively large (just discernible in the light microscope) crystals were obtained Although the particle size was still quite small surface variation was minimized and, because of the elevated temperature and aging the presence of defects would also be minimized. Under these conditions, a whole series of hydroxy apatites was prepared, with Ca/P ratios varying from 1.3 to over 2 0 Their results were best described by assuming a limited substitution of $2H_3O^+$ for $1Ca^{++}$ with a maximum of 4 hydronium ions per unit cell. A similarly substituted unit cell has

been proposed by Arnold (1). For example, Arnold's octocalcium phosphate $[Ca_4^{++}H^+(PO_4^{\equiv})_3 \cdot 3H_2O]$ multiplied by 2 can be written



$$[Ca_8^{++}(PO_4^{\equiv})_6(OH^-)_2 \cdot (H_3O^+)_4]$$

from which the general formula for the unit cell of hydroxy apatite becomes



$$[(Ca_{10-x}^{++})(H_3O^+)_{2x}][(PO_4^{\equiv})_6(OH^-)_2],$$

where x may vary from zero to a maximum of 2.

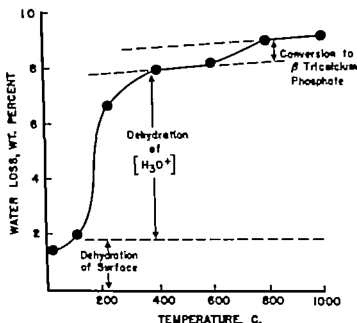


FIG. III 3.—Typical data from Kunin (21) showing the loss of water as hydroxy apatite is heated. The specimen was initially dried in a desiccator, then heated at each temperature to constant weight.

One of the principal reasons that this general formulation is attractive is that it explains the water loss observed on dehydration by heat. A typical dehydration curve (21) is given in Figure III 3, where it is seen that the water loss is stepwise. After a small initial loss of presumably surface-adsorbed (36) water, a large loss occurs between 110° and 400° C. No measurable changes in structure take place, however, until about 800° C. The diffraction patterns may sharpen from crystal growth, but the big change is seen when the calcium-deficient lattice collapses with the loss of hydroxyl ions, to give an X ray diffraction

pattern of β-tricalcium phosphate, $Ca_3(PO_4)_2$. If the water driven off between 110° and 400° C is assumed to be due to the dehydration of H_3O^+ it is simple to predict from the Ca/P molar ratio just what this water loss should be. A comparison between prediction and observation is given in Figure III-4 The agreement between theory and fact is good and since none of the other structural concepts offers any explanation for such a great water content, this can be considered evidence for the lattice substitution of $[H_3O^+]$ for calcium Furthermore as shown later (chap iv), the hydronium ion can displace the calcium ion on the *surfaces* of the crystals. It seems only reasonable to suppose then that *certain* calcium positions, probably some of the column positions (1) within the lattice, may be replaced but there is a limit to the number of calcium ions which can be displaced. No one has been able to obtain calcium phosphate precipitates having a Ca/P ratio of less

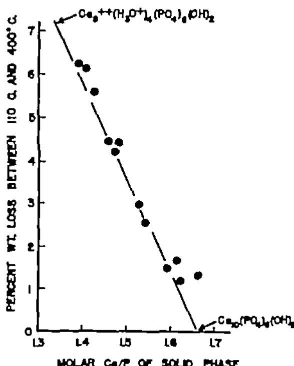


FIG. III-4.—A plot of the relation between water lost on heating and the Ca/P ratio redrawn from Kunin *et al* (21) The line represents the theoretically expected loss, assuming that (*a*) $2(H_3O^+)$ replaces $1Ca^{++}$ (*b*) all surface-held water is lost below 110° and (*c*) (H_3O^+) converts to H plus water vapor between 110° and 400° C

than 1.33 free of contamination with secondary calcium phosphate $CaHPO_4 \cdot 2H_2O$ Thus a maximum of 1 in 5 of the total calcium positions can be substituted and many of these are undoubtedly surface positions.

It is difficult to see how a substitution of even a small percentage of the internal calcium positions by hydronium ions could occur without causing measurable changes in the unit cell dimensions.[1] Calcium has an ionic radius of about 1 A while H_3O^+ ions are of the order of 1 4 A or greater (41) Indeed Carlström (8) has discussed the appearance in crystals of a low Ca/P ratio of additional fairly well-defined reflections corresponding to interplanar spacings which could not be identified with those of any calcium phosphate so far investigated." These were also observed in diffraction patterns of the octocalcium phosphate" of Arnold (1) Further discussion of this problem will be found in chapter IV under Heteroionic Exchange."

INTERNAL LATTICE DEFECTS

Apart from such ion substitutions as may occur within the lattice it is safe to conclude that most unheated unaged preparations of hydroxy apatite represent imperfect crystals with many defects present throughout the lattice. The fact that thermal reconstruction or recrystallization of hydroxy apatite crystals occurs spontaneously is one line of evidence (34) Another pertinent finding is the sharpening of diffraction patterns by heating crystals at 400° C a temperature at which crystal fusion would not occur

There is no argument against the occurrence of defects. The important question is How do defects account for variable stoichiometry? If we presume that a particular preparation exhibiting a low Ca/P ratio is defective in calcium i.e. has many calcium positions vacant, we are confronted with the problem of electrostatic imbalance It is perfectly reasonable to picture holes in a lattice structure, but it is necessary to leave out both cations *and* anions. If one leaves out calcium while retaining all the phosphate ions, some other cation must be added. Water and hydrogen ions have been suggested as possible candi

1 It is for this reason that care has been taken to avoid the use of the term "isomorphic substitution." This term implies that no structural change is involved. Hendricks has argued that the substitution of H_2O or H_3O^+ for Ca should increase the index of refraction, while actually the index of refraction of low Ca/P preparations is lowered (16) His argument assumes an *isomorphic* substitution and the applicability of a method which probes at the "level of 1 000 to 10 000 atomic dimensions (16) in crystals which are only 30-50 atomic dimensions thick.

dates for balancing charge (38) but this concept is the same as the concept of lattice substitution given above An alternative is to postulate that the internal lattice which is defective in one ion gives the crystal a net charge which is balanced by a surface-adsorbed layer of ions of the opposite charge. For example, hydroxy apatite precipitated from a solution containing *excess* calcium might be deficient in phosphate and adsorb hydroxyl ions to maintain neutrality The converse can also be pictured.

While defects are known to be present, then it is possible to explain variable stoichiometry by *internal substitution* and *surface adsorption* alone

SURFACE REACTIONS

Surface exchange and surface adsorption are plausible explanations for variable stoichiometry because the crystallites are so small that the specific surface is truly enormous—from 10 to over 200 M^2/gm. While the crystal size is variable, the best electron microscope pictures indicate that bone crystals and many synthetic preparations are only 2–4 unit cells thick one-half to two-thirds of the unit cells are located in the surface and possess one or more unshared sides. Thus variations in surface composition can significantly affect the composition of the whole preparation as determined by chemical analysis.

Carlström has made calculations which illustrate clearly how variations in surface composition can explain the variable stoichiometry observed (8) His graphic summary of these calculations is illustrated in Figure III 5 Surface variations, then *can* account for nearly all the variable stoichiometry of *unheated* apatite preparations. There are two studies however which indicate that the internal lattice also is somewhat variable

Olsen (37) determined, by means of $P^{32}O_4$ the exchangeable or surface phosphate of a number of hydroxy apatite preparations whose specific surface areas varied from 14 to 58 M^2/gm He found an average of 0 238 mg phosphorus/M^2 of surface irrespective of specific surface area or Ca/P ratio! We have determined the surface Ca/P ratio on two apatite preparations by measuring both exchangeable calcium and phosphate using Ca^{45} and $P^{32}O_4$ (33) These results are given in Table III 2 and indicate that the lack of stoichiometry is *shared* between the surface and the lattice interior the surface showing the larger deviations in Ca/P ratio In fact "L-apatite" having an over-all ratio Ca/P

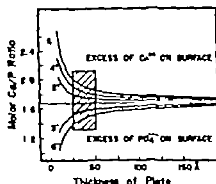


FIG. III 5 —"The relationship between the particle size and the Ca to P ratio when the particles are assumed to be plates with infinite extension in two dimensions. This simplification is justified by the experimental data reported and gives the *minimal* surface area. It is further assumed that each free unit cell surface can carry an excess of one two or three Ca-ions or one or two phosphate groups. The hatched area in the figure covers the particle sizes of the apatite crystallites in bone and most synthetic precipitates as they commonly appear" (taken from Carlström [9]) The thickness of the plate should be multiplied by 2 to give the diameter of a corresponding prism of infinite length.

TABLE III 2

COMPARISON OF COMPOSITION OF TWO APATITE PREPARATIONS

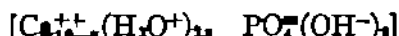
	Weight Per Cent	
Apatite	"31"	"L"
Total Ca	36 0	38 0
Total P	18 8	17 7
Surface Ca	4 42	4 52
Surface P	6 41	5 47
	Molar Ca/P Ratios	
Apatite	"31"	"L
Total	1 48	1 67
Surface	1 02	1 38
Interior	1 51	1 68

These synthetic apatites, named 31 and "L for no significant reason, have been well characterized and extensively studied in our laboratory as model systems. They will be referred t number of times in subsequent discussions

of 1 67 [theoretical for $Ca_{10}(PO_4)_6(OH)_2$] exhibited a calcium-deficient surface, a Ca/P ratio of 1.38.

When the apatite crystals are placed in solutions containing ions foreign to the lattice but capable of substituting for calcium, phosphate, or hydroxyl ions, surface reactions become dominant. Such surface exchange substitutions are especially important in determining the composition of bone mineral and are discussed more fully in chapter IV

Summarizing present information on the crystallography of the hydroxy apatites, we may conclude that there exists a family of materials, all of which exhibit an X ray diffraction pattern (and therefore a structure) similar to fluorapatite, [$Ca_{10}(PO_4)_6 \cdot F_2$] The stoichiometry of these materials is quite variable, exhibiting molar Ca/P ratios of from 1.3 to over 2. Hydroxy apatite preparations are always microcrystalline and present an enormous surface variations in surface composition appear to account for most of the variations in the over-all Ca/P ratio of unheated specimens. Many defects also occur within the lattice, and perhaps H_3O^+ may substitute for the calcium ion to a limited extent[7] as indicated by the general formulation



$$[Ca^{++}_{10-x}(H_3O^+)_{2x} \cdot PO_4^{\equiv}(OH^-)_2]$$

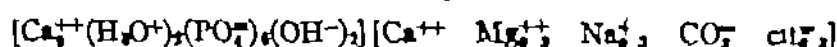
It would appear that the area of ignorance concerning the crystallography of hydroxy apatite involves an evaluation of the relative contribution of internal substitution, defects, and surface reactions in producing variable stoichiometry It would be much less confusing if investigators refrained from giving names to isolated preparations. The terms octocalcium phosphate," a tricalcium phosphate hydrate," "pseudo-apatite," etc. have little or no meaning and, as labels, give the false impression of describing true, stoichiometric compounds. To describe a preparation of hydroxy apatite one requires not a term but a body of analytical data chemical composition, including trace contaminants water content and dehydration curves X ray diffraction data surface-area measurements and a measure of surface composition by $P^{32}O_4$ and Ca^{45} exchange plus electron-microscope pictures and solubility data.

HYDROXY APATITE CRYSTALS OF BONE

We have discussed the importance of surface substitutions in affecting the stoichiometry of apatite preparations. In physiological fluids there are many ions present which are capable of substituting for calcium or phosphate, particularly in the surfaces of the crystals of bone

It is not surprising to find then that bone mineral is a quite impure hydroxy apatite having as its principal impurities carbonate, 6 per cent citrate 1 per cent sodium, 0 7 per cent, and magnesium 0 7 per cent, with traces of fluoride. Synthetic apatites mirror the composition of fluids in which they are placed (35) If precipitates of calcium and phosphate are caused to form in solutions approximating the composition of serum the resulting products show approximately the same composition as bone mineral (12 22) Apparently there is nothing magical about the presence of the impurities in bone. They are there as a passive physicochemical consequence of the presence of these ions in the fluids in which the crystals form (cf chap iv)

There have been many attempts to describe bone mineral as a *mixture* of insoluble salts. However there has been no convincing evidence brought forth to indicate the presence of more than one phase. Furthermore, because the body fluids are undersaturated with respect to such salts as $CaCO_3$ Ca_3 (citrate)$_2$ $MgCO_3$ $NaHCO_3$ etc., it is foolish to postulate their existence in bone No one can be dogmatic about the exact nature of the bone mineral, but the most logical view at present is that bone mineral consists of microcrystals of hydroxy apatite whose composition is determined largely by surface exchange (17 35) and to some unknown extent by internal defects and substitutions. If these impurity percentages are converted to mole ratios, the over-all composition of average bone mineral may be written thus



It is seen that some of the calcium is required to neutralize the charge of the anionic impurities, principally carbonate The residual Ca/P ratio is thus 1.5 considerably less than the theoretical value, 1 67 Bone mineral then, is one of the series of apatite minerals which are calcium-deficient. A moment s consideration will show that this member of the apatite series, Ca/P = 1.5 is advantageous from the standpoint of the animal s well-being. An apatite of this ratio can undergo variations in *either* direction with minimal changes in the size and character of the crystals. This permits the animal under extremes of dietary stress, to lay down a satisfactory functional bone salt.

CRYSTAL MORPHOLOGY OF BONE

It has long been known that the crystallites of bones and teeth are extremely minute. Since they are too small to be seen in the light microscope it was by X ray scatter that we first learned how very tiny

they are—of the order of a few hundred A. While X ray diffraction, low-angle X ray scatter and similar techniques have served and will continue to serve as useful tools in this field, our best hope for the delineation of crystal morphology is the electron microscope.

With a theoretical resolving power of less than 1 A, design development of the electron microscope and sectioning and mounting techniques have improved so rapidly that the effective resolving power has dropped to 10 A while 3 A is a foreseeable goal and 1 A is a reasonable hope in limited applications (10) Such resolution is in the realm of *atomic* dimensions and the investigator can at least dream of probing into the *inner* structure of the individual crystal! Surely before many years have passed there will be final answers concerning the size, shape and variations in crystal morphology These answers will not come easily Only part of the difficulty is in instrument construction. lens design, current stability thermal stability etc. Great problems develop in sectioning ultra thin specimens, light elements give extremely poor contrast carbon deposition on the specimen blots out detail and as resolving power improves, it is with increasing difficulty that the electron microscopist can discriminate between the real and the artifact. At the present time, much of the electron-microscopic findings on bone structure can be considered the fumbling beginnings of a new science

There is, as yet no unanimity of interpretation among the several groups investigating the crystal morphology of bone. Viewing intact sections of bone in the electron microscope, Robinson and Watson (40) describe minute tablets of a few hundred A in length and breadth with a thickness of only a few unit cells, 20–50 A Finean and Engström (13) interpret their low-angle X ray scatter diagrams to mean that bone crystals are rods or hexagonal prisms about 200 × 75 A. Ascenzi (2) has concluded from his electron-microscope studies that the bone mineral is a *continuous* phase with small "spheroids of entrapped organic substance We hope that, before long this confusion will be resolved. At present, however we can only compare and criticize these three schools of interpretation.

Of the three proposals, that of Ascenzi (2, 7) supported by the electron micrographs of Barbour (4) is the most unique and surprising The mineral of bone is described as a continuous solid phase of honeycomb structure The spaces within the honeycomb are thought to be occupied by organic material—the ground substance. Ascenzi claims that the ground substance itself in the absence of mineralization shows

a micellar structure Robinson's interpretations are criticized on the basis that artifacts were introduced in the pretreatment of the bone samples and sections taken for electron microscopic study (2) Ascenzi criticizes (6, 7) Engström's interpretation of low angle X ray scatter diagrams because theoretically the same diagram could be given by the suggested honeycomb structure (6, 7) According to Ascenzi it is the micelles of organic substance in the honeycomb structure which give the pattern and dimensions of Engström's inorganic rods or prisms 200 × 75 A

Ascenzi's criticisms of the proposals of Robinson and Engstrom can in fairness, be said to apply also to his own work. For example every one recognizes the ease with which artifacts can be introduced into an electron-microscopic study (14) Ascenzi himself employed HCl HNO_3 2 per cent KOH in glycerin and trypsin digestion for his specimens. While it is true that Engström's interpretation of the low angle X ray scatter diagrams is not *exclusively* correct neither is Ascenzi's.

Ascenzi's interpretations raise many questions that are difficult to answer Mineralization of osteoid begins with the aqueous-organic medium as the continuous phase It is possible, but unlikely that these two phases could invert i e. the mineral particles become continuous while the aqueous medium becomes coagulated into tiny "spheroids. Certainly in the absence of organic material but in aqueous suspension hydroxy apatite forms as minute crystals, not as a continuous structure. Organic material would be expected to prevent coalescence of the inorganic particles, not to induce crystal agglomeration at a distance by a self-aggregation of the organic matter into spheroids. Furthermore, were the mineral phase the continuous one the specific surface area of ashed bone preparations would be expected to increase upon being ground. Actually bone ash exhibits a constant specific surface independent of particle size (17 32) Finally it is not clear where the collagen fibers are located in Ascenzi's honeycomb The bulk of the organic material in bone is collagen the fibers of which do not correspond to the micelles of 200 × 75 A How does the honeycomb structure explain the co-orientation between the *c*-axes of the crystals and the longitudinal direction of the collagen fibers? It seems possible that Ascenzi's honeycomb structure is more apparent than real and that the dense, continuous phase actually possesses a finer particulate structure not yet made visible because of poor resolution For example in Figure III-6 is presented a portion of an electron micrograph of a purely inorganic, synthetic hydroxy apatite preparation This material cannot

possibly contain organic spheroids yet the unresolved agglomerates of crystals give something of the appearance of Ascenzi's "honey comb"

The chronological development of Robinson and Watson's views of a tablet-shaped crystal follows a consistent pattern. The first preparations were autoclaved and "blended." These showed crystallites around



FIG. III-6.—An electron micrograph of the L-apatite

500 A in length. Later having shown that glycol-ashing caused growth of the crystals, they examined intact sections of bone which had received no more drastic treatment than osmic acid fixation at pH 7 4 at room temperature. In these unheated specimens the crystals appeared to be smaller but still showed a tabular habit. While it is true that manipulative techniques, particularly heat, can alter the crystal habit, it is hard to conceive of any great alteration of preformed crystals re-

sulting from such a mild fixation procedure For example Weikel (43) has shown that preformed crystals of hydroxy apatite maintained the same specific surface 68 M²/gm throughout a one month equilibration (with stirring at room temperature) in buffered saline

Robinson (39) currently describes the hydroxy apatite crystals of bone as thin tablets 200 300 A in length and breadth with a thickness of only a few unit cells, 20 50 A The crystals are closely associated with the collagen fibers the c axis and the longitudinal direction of the fibers being approximately parallel The crystals are located principally between the major 610 A spacings of the fibers. In dentine at least the crystals are probably located *within* the fiber bundles (42) If this is true also of bone the removal of all organic material would leave a conglomerate of tiny crystals, which if not resolved by the microscope might give the appearance of the "honeycomb" seen by Ascenzi.

Finean and Engström (13) and Carlström (8) have relied principally on low-angle X ray scatter to deduce the dimensions and orientation of the bone crystals. They have concluded that the crystals are fairly symmetrical in cross-section, of a dimension in the range 40–75 A In length they have reported dimensions of 200 and 220 A. They established that the crystalline c axis parallels the longitudinal direction of the collagen fibers in oriented specimens. They suggest that three rods correspond to the 610 A spacings of the collagen fibers.

The problem of rods versus tablets is difficult to evaluate Watson and Avery (42) have on occasion, seen rod-shaped images which were interpreted as tablets on edge. Finean and Engström (13) have, on occasion, seen tablets which appeared to be aggregations of rods. Engström has suggested (11) that an aggregate of three rods would give a tablet of the dimensions described by Robinson and Watson. Since the analysis of low-angle X ray scatter diagrams depends in a large measure upon the assumptions employed no final conclusions can be given.

From the present status of information on the crystals of bone mineral it seems reasonable to suppose that some of the divergence among investigators is caused by experimental artifacts. Some may also come from the examination of specimens in different stages of maturation. Thus a section beginning to mineralize would be expected to show collagen fibers associated with small isolated, fragmentary crystals. As mineralization continued to the exclusion of interstitial water (cf chap x) the growth of crystals within the fibers and epitactic overgrowth (cf. chap vii) of pre-existing crystals would give the appearance of an almost continuous mineral phase

Whatever the final conclusions may be, certain general inferences already seem certain. The unit crystal of bone mineral is extremely small of the order of 200 by 30–70 A A tentative picture, subject to change of a 'typical bone crystal is given in Figure III 7 Such tiny

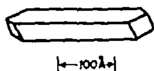


Fig. III 7 —A diagrammatic picture of a "typical" bone crystal according to Robinson and Watson. Note that this tiny colloidal structure contains only about 2,500 unit cells, giving it a "molecular weight" of about 2½ million.

particles present enormous surfaces for exchange substitutions of ions foreign to the hydroxy apatite lattice. These surface reactions render the bone mineral quite variable in composition and solubility

CHAPTER IV

Surface Chemistry

The chemistry of bone mineral is almost entirely a problem in surface chemistry From its initial seeding induction through its growth maturation, and dissolution the tiny crystal must interact with its fluid environment by ion interchanges across a solid solution interface.

Modern civilization in large measure depends on surface properties brake linings, powder metallurgy catalytic cracking and Scotch brand tape are a few examples of products and processes dependent on surface phenomena and although we have skilfully harnessed surface properties, much of our success is the result of luck and intuition" (22) The fundamentals of this field are only beginning to fall under concerted research attack. In particular the solid surface–solvent interaction of ionic crystals and aqueous solutions has been sadly neglected. Therefore, we must examine the existing information on the surface chemistry of the bone mineral against a woefully inadequate theoretical background. This means that most of the interpretations to be given are tentative and perhaps, temporary

The Magnitude of the Surface

We have described the crystals of bone as flat tablets or rods a few hundred angstroms in length and breadth and only a few unit cells thick (30–70 A) From these dimensions and the density (about 3) of bone mineral it is possible to calculate the specific surface in M^2/gm A series of such values calculated for hypothetical crystals of various sizes has been assembled in Table IV 1 They show clearly that it is the *thickness* dimension that primarily determines the specific surface Thus, decreasing the length fifty fold increases the surface only about 5 per cent (1 versus 3) while decreasing the thickness threefold (5 versus 3) increases the surface two and a half times. It is thus very dimension—the thickness—that cannot be estimated with any degree of certainty with the present resolving power of the electron microscope. If we were only certain that *all* the crystals of bone were tablets or rods, we should be able to determine the average thickness of the crystals directly by measuring the specific surface of the mineral phase of bone

It is impossible to obtain a surface-area measurement of intact bone crystals (by ethane or N_2 adsorption) because of the presence of water and organic matter Unfortunately no one has yet devised a procedure for removing the water and organic material without inducing possible alterations in the habit and size of the crystals. There are four common procedures (*a*) dry ashing above 400° C which is certain to induce crystal growth (52) (*b*) alkaline-solvent ashing (KOH in either glycol or glycerin at 200° C) which probably alters crystal size (*c*) steaming of bone which is not certain to remove all organic matter and, finally

TABLE IV 1

VARIATIONS IN SPECIFIC SURFACE WITH VARYING DIMENSIONS OF HYPOTHETICAL CRYSTALS (CALCULATED)

Crystal	Length (A)	Width (A)	Thickness (A)	Specific Surface (M^2/Gm)
1	10 000	200	10	670
2	500	300	10	700
3	200	200	10	700
4	200	200	20	380
5	200	200	30	280
6	300	200	30	270
7	300	200	70	150
8	500	300	100	100
9	Rodlike prism 210×70			220†

Range of sizes according to Robinson and Watson (cf chap ii)
† According to Engström and co-workers (cf chap iii)

(*d*) refluxing of bone with ethylene diamine vapors which removes organic matter efficiently but has not been studied with respect to its effect on crystal habit. For these reasons, it is questionable whether there exist, at present *any* valid measurements of specific surface for unaltered bone mineral. Published values vary from a minimum of 66 M^2/gm for steamed beef bone (26) to a maximum 244 M^2/gm for glycol-ashed rabbit bone (40) We can only guess, then at the surface area of unaltered bone crystals. Undoubtedly it is at least 240 M^2/gm and probably much more—perhaps over 300 M^2/gm.

It is interesting to note that the rod structure proposed by Engström s group for the bone crystal would have a specific surface of 220 ± 50 M^2/gm Robinson and Watson s proposed tablets would also exhibit a specific surface in this same ra 300 M^2/gm On the basis of the most reliable available meas specific surface and

crystal dimensions, we can deduce that the crystals of bone have an average thickness of only 30 A, or about 3 unit cells thick! It is not surprising then that the chemistry of bone mineral is a problem in surface chemistry However we must keep in mind that we are dealing with average values. It is unlikely that the crystals of bone are uniform. More likely in any one sample of bone the crystals range in size from very tiny aggregates to crystals larger than the average value deduced from these surface-area measurements.

THE HYDRATION LAYER

For many years physicists have accepted the view that a solid surface when exposed to a liquid takes on an extremely thin film of bound solvent the immobile layer " The thickness of this film is not known with certainty but hovers around an estimated mean of about 100 A. The nature of the bonding is not clear (6) The surfaces of hydroxy apatite crystals, too take on a solvent layer and because the specific surface of these crystals is so large the total amount of solvent bound is surprising When the solvent is water the amount held is almost unbelievable. But the extent of solvation or hydration depends, in large degree, upon the method of measurement Thus, if the crystals are exposed to water as a vapor an adsorption isotherm is obtained (46) This isotherm can be analyzed mathematically by BET theory (9) to give a calculated monolayer" the surface area so deduced corresponds to the surface area obtained by ethane adsorption. The maximum amount of hydration adsorbed from the vapor phase is four times the calculated monolayer If on the other hand, the crystals are exposed to liquid water and then subjected to an initial, mild centrifugation to remove all but the 'hydration layer the amount of bound water remaining varies inversely with the force of subsequent centrifugation up to 30 000 $\times g$ after which it cannot be reduced further (39) In all cases, this irreducible minimum amounts to a great deal more water *held* by the crystals than is maximally *adsorbed* from a vapor phase (cf. Table IV 3) This suggests that the surfaces of crystals suspended in aqueous buffer differ structurally from the surfaces of crystals in the dry state. Apparently when in contact with bulk water the crystals form an electric double layer ("Helmholz double layer" or "zeta potential") which in turn, binds the 'immobile solvent layer" ascribed by the physicists to all solid surfaces. The fact that the crystals immobilize such a large quantity of water is itself good evidence for the presence of a strong electrical field at the crystal surface.

According to a qualitative concept developed by Weyl (66) the origin of the field is the electrical asymmetry of the surface. Thus, in the crystal interior each cation is completely surrounded by a restraining field of anions and conversely, each anion is surrounded by cations. At the surface however the ions are not completely shielded. Rather there is an interface of residual electric charge from each surface ion projected into space (or into the solvent) as a mosaic patchwork of positive and negative fields. This may be illustrated diagrammatically

									Interface	
Crystal Interior									Surface	Space or Solvent
+	−	+	−	+	−	+	−	+	−	⟶
−	+	−	+	−	+	−	+	−	+	⟶
+	−	+	−	+	−	+	−	+	−	⟶
−	+	−	+	−	+	−	+	−	+	⟶
+	−	+	−	+	−	+	−	+	−	⟶
−	+	−	+	−	+	−	+	−	+	⟶

Weyl presents four ways in which the charge asymmetry can be diluted out to reduce the surface energy "(i) polarization of the surface ions (ii) increasing the number of most polarizable or decreasing the number of least polarizable ions in the surface (this change produces crystals with excess electric charges) (iii) advancing the most polarizable ions into the extreme outer layer and retracting the least polarizable ones (this distortion produces an electric double layer and must have a depth action) and (iv) chemisorption of molecules or ions" (66) Applying these principles to the tiny platelets of hydroxy apatite, we find that methods ii and iii are eliminated by the size of the crystals and the nature of the ions present Calcium ions are virtually non-polarizable, and phosphate ions are of course, polarizable Any increase in phosphate ions would set up strong negative fields on each face of the crystals, and, separated by only 30 A, these fields would interact strongly (24) Thus, though not stated by Weyl the "defect mechanism, ii, requires depth action and is eliminated Mechanism iii is also eliminated by the depth-action requirement, leaving methods i, which can be satisfied in part by polarization of phosphate and iv chemisorption.

Unfortunately for us, Weyl does not clearly define his meaning of the term chemisorption. This catch-all term has many meanings, some of which would not offer an explanation of how the surface energy at the interface is reduced In its simplest form chemisorption could be pictured as an adsorption of a layer of ions of charge opposite to the mosaic of excess surface charge. This, however, merely creates a new

surface itself presenting a mosaic of residual charge If the ions in the adsorbed layer were not polarizable there would result no net decrease in surface-charge asymmetry If the adsorbed ions were polarized there would be only a partial lowering of the surface energy The facts are clear To reduce the charge asymmetry at a crystal-solution interface successive layers of polarizable cations and anions are required which project outward into the solution until electroneutrality is established This can be visualized as analogous to a "double Helmholz double layer where the surface predominantly electropositive because of the polarizability of the phosphate ion but also exhibiting a mosaic of residual negative valence binds successive layers of polarized phosphate ions and hydrated calcium ions which gradually dilute the surface electrical asymmetry Ionic calcium in the unhydrated, crystalline state is non-polarizable but is quite polarizable by virtue of its layer of oriented water molecules in the hydrated state (Ca^{++} $10H_2O$) This interface of hydrated calcium and polarized phosphate ions is pictured diagrammatically in Figure IV 1

Dilution of the surface charge by bound ion layers is possible only in a medium of high dielectric constant, such as water The depth requirements not possible within the thin crystal are thus met by this bound ion and solvent layer—the hydration shell The ions in the hydration shell are not fixed and motionless but are mobile and reactive only the electrostatic field which is responsible for the origin of the hydration shell is fixed and static. When the crystals are in a solution containing polarizable ions other than calcium and phosphate, there will be many substitutions and replacements in the hydration shell by ions which are normally foreign to the crystal lattice itself This process, ***heteroionic exchange*** is developed more fully subsequently

The presence of a bound-ion and solvent layer around the crystal may set up a diffuse, oriented layer of ill-defined dimensions extending farther out into the solution. This second solvent layer if such exists, is very weakly held and can easily be separated from the hydration shell itself Thus, if an aqueous suspension of hydroxy apatite placed on a membrane, is subjected to increasing centrifugal force the bulk solution is removed at forces below $10\,000 \times g$ From 10 000 to $30{,}000 \times g$ the weakly bound layers are gradually removed leaving only the crystals and their hydration shells. These data are illustrated in Figure IV 2 showing a minimum hydration of 35 mM/gm of L-apatite. This, on a volume basis, means that every crystal binds a hydration shell 1 9 times its own volume!

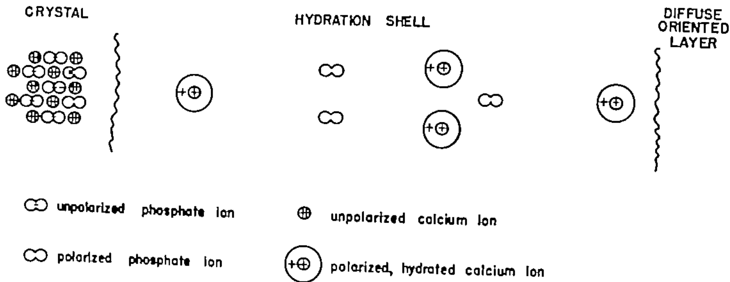


FIG. IV 1.—A diagrammatic representation (ignoring OH^-) of the hydroxy apatite crystal solution interface. The quantitative relations between crystal and bound ions as represented here have been established experimentally and are listed in Table IV 2.

From these considerations and from additional surface data assembled in Tables IV 2 and IV 3 we may tentatively picture an individual crystal of hydroxy apatite in aqueous suspension as in Figure IV 3 In presenting this suggested space model of the crystal and its interface, we must emphasize certain points. First the whole construction has been based on studies of a single synthetic hydroxy apatite preparation. Quantitative relationships may vary with different samples of

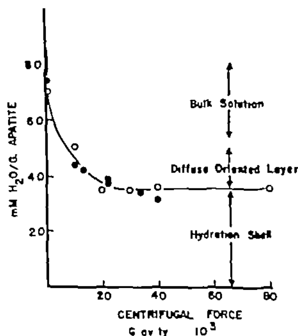


FIG. IV 2.—Data illustrating the removal of water from hydrated crystals by high-speed centrifugation Solid circles taken from Neuman *et al* (46) open circles from Myers (39)

hydroxy apatite. Second, on the basis of water-binding, a five-compartment system can be delineated on the basis of ion composition and ion exchange, only four compartments can be differentiated. The bulk solution and the diffuse oriented layers are essentially the same (46) Since our primary concern is with ion exchange, we shall represent hydroxy apatite crystals in aqueous suspension as a four-compartment system (S_1 and S_2) the bulk solution plus the diffuse oriented layer (A) the hydration shell (B) the crystal surface and (C) the crystal lattice interior According to this construction, S_1 and S_2 have the same composition The hydration shell (A) will mirror to a limited extent the com

TABLE IV 2

COLLECTED SURFACE DATA ON L-APATITE

Dimensions of crystals Specific surface Dimensions of unit cell No. surface unit cells	1,000×500×150 A 67 8 M^2/gm 6 88×9 42 A 1×10^{20}/gm	Ref 41

	mM/Gm	Molecules/ Surface Cell	Ref.
H_2O monolayer* by vapor adsorption	1	6	46
Hydration shell (centrifugation at 80,000×g)	35	210	39
Hydration shell and diffuse layer	44	264	46
Calcium in hydration shell*	0 2	1 2	38
Calcium in surface	0 15	1	38
Phosphate in hydration shell	0 2	1 2	57
Phosphate in surface	0 4	2 4	57

* In KCl solution, μ = 0 16, pH 7 2, T = 37° C., at solubility equilibrium [Ca] ≃ 0 35 mM/L, [P] ≃ 0 16 mM/L Values for calcium and phosphate in the surface are approximate.

TABLE IV-3

APPROXIMATE CALCULATIONS OF THE HYDRATION OF L-APATITE

Water Held as	Hydration Shell		
	Weight (Mg/Gm)	No. of Molecular Layers	Film Thickness (A)
Adsorbed monolayer, V_m†	17 6	1	3
Hydrated surface calcium (Ca 10H_2O) (28)	52	3	10
Highest value adsorbed from vapor phase	78	4	13
Minimum retained (centrifugation at 80 000×g)	540	60	60
Maximum retained (centrifugation at 10 000×g)	800	95	95

* Assuming a volume of 31 A^3 for H_2O molecule.

† The monolayer is that defined mathematically by the V_m of the Brunauer Emmett Teller equations for gas adsorption (9)

position of the bulk solution (S_1) There would be one major difference, however layer (4) must contain a high concentration of neutralizing multicharged polarizable ions, principally hydrated calcium and polarized phosphate ions. For example the data in Table IV 2 indicate that in pure aqueous KCl-hydroxy apatite suspensions, the concentration of both calcium and phosphate in the bound hydration layer was approximately 0 6 molar while the concentration of these ions in the bulk solution was only about 0 0002 molar

While, these considerations of crystal-solution interfaces probably also apply to all ionic crystals the importance of the interface becomes less and less with increasing particle size. Since hydroxy apatite crystals are uniquely small the interface is uniquely important in determining the chemical properties of this mineral There does exist in nature an

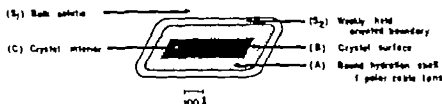


FIG. IV 3—A diagrammatic representation of a cross-section view of hydroxy apatite crystal in aqueous suspension.

other mineral group containing non-polarizable cations and exhibiting a tabular crystalline habit of extremely small dimension. This group—the clays—also exhibits unique surface properties, with heavy hydration and great ion-exchange activity Though of markedly different chemical composition the clays and bone mineral have many physicochemical properties in common (28)

ISOIONIC EXCHANGE

The *entire* chemical behavior of the mineral of bone involves the transfer of ions to and fro across the hydrated crystal-solution interface. In the broadest sense, this could logically be considered *ion exchange* By convention, however the term "ion exchange implies no net gain or loss between the interacting phases. Therefore, in the subsequent discussion we shall accede to convention and reserve the term ion exchange to applications wherein the solid and liquid are essentially at equilibrium Where there is a net transfer of ions from solution to the solid phase, the terms *mineral acquisition* or *crystal growth* will be used. The term *dissolution* will be used to describe a net transfer of ions

from solid to solution It should always be kept in mind however that all three processes involve the same basic underlying mechanism. Thus a crystal undergoing dissolution is both adding ions to and losing ions from its surface, but the rate of loss exceeds the rate of gain

Of the three main ionic constituents of the hydroxy apatite lattice, convenient radioisotopes are available for two $P^{32}O_4^{\equiv}$ and Ca^{45++} Though heavy isotopes for oxygen and hydrogen and radioactive tritium are available for labeling the hydroxyl group, the rapid formation of water renders impractical the study of hydroxyl exchange in an aqueous suspension of crystals.

In the subsequent section the exchange of the normal lattice ions—calcium and phosphate—for their radioactive counterparts is termed *isoionic exchange* When different ions are involved, i.e. various cations displacing calcium various anions displacing phosphate or hydroxyl groups, the term *heteroionic exchange* will be employed.

PHOSPHATE EXCHANGE IN A MODEL SYSTEM

We have by definition limited the use of the term "ion exchange" to the crystal-solution system under equilibrium conditions. Only rarely have exchange studies of apatites or bone mineral been carried out under equilibrium conditions. This requirement makes it necessary that a minute volume of carrier free isotope be added to an aqueous-crystal system which has already achieved equilibrium conditions with respect to all ions of the solid and the solution. The high cost of *carrier free* calcium has precluded an adequate study of calcium exchange. The heterogeneity in size and composition of bone crystals necessitates the use of a synthetic model. A fairly thorough investigation of the phosphate exchange of L-apatite has been made (65) however and this will be reviewed in some detail

Apparatus and Methods

The apparatus employed was modeled after Schweitzer and Nehls (53) and is given in Figure IV-4 Because of the difficulty in removing colloidally dispersed crystals, care must be exercised in separating the two phases. Three methods have proved successful (*a*) centrifugation at 80 000 × *g* (*b*) filtration through selected glass filters of pore size of less than 5 μ (*c*) filtration through millipore filter membranes, which has proved to be the most convenient.

The solutions were weakly buffered with 5 mM/l of diethyl barbituric acid brought to pH 7.2–7 4 with 2 mM/l of KOH. They con-

tained, in most cases, 5 µg P/ml and 10 µg Ca/ml (just saturated) and KCl to give $\mu = 0.165$. To 700 ml of this solution at 37° C was added 1 gm. of L apatite. The crystal suspension was stirred for at least 24 hours, which was shown to be sufficient time to establish equilibrium between solid and solution. Then 1 ml. or less of carrier free radio phosphate (P^{32}) solution was added. The level of radioactivity was previously adjusted in this aliquot to give on final dilution approximately 1 000 counts per minute per milliliter in the suspension

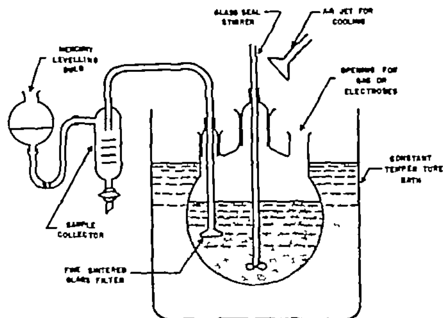


Fig. IV-4.—Modified apparatus of Schweitzer and Nebls used in the study of the kinetics of radioactive exchange (53)

liquid. Because the exchange reaction is very rapid it was necessary to determine the initial level of radioactivity by a separate dilution of an identical aliquot of P^{32} to 700 ml, with the buffer solution containing no crystals. The course of the exchange was followed by periodic removals of 1 ml. samples of filtrate through the sampling tube. Each sample was then assayed for radioactivity. A final large portion of filtrate was removed to provide for the accurate analysis of total concentrations of calcium and phosphate at the solubility equilibrium. By means of this technique the addition of labeled phosphate and the removal of small volumes for analysis did not alter significantly the solid-solution ratio or the equilibrium condition already established

Kinetic Analysis

In terms of the space model just developed for the hydroxy apatite crystal Figures IV 1 and IV 3 we should expect the over-all exchange reaction to involve three successive transfers of labeled phosphate between the four distinct compartments (1) from the bulk solution to the hydration shell (2) from the hydration shell to the crystal surface and (3) from the crystal surface to successive layers of the internal lattice by intracrystalline exchange.

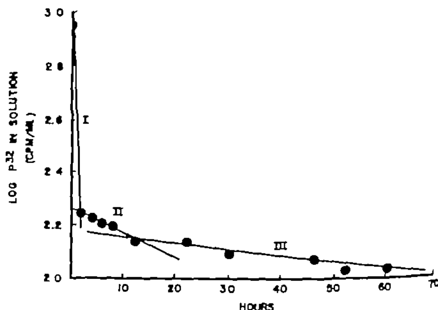


FIG. IV-5 —Data illustrating that the over-all exchange process occurs as a series of reactions exhibiting first-order kinetics.

The disappearance of radiophosphate from solution does indeed take place in three separate, successive steps. Govaerts (23) has also reported a three step exchange process. The three steps are not readily apparent when the radioactivity of the solution phase is plotted against time. When, however the logarithm of the solution activity (cpm/ml) is plotted versus time, there can be seen a series of three lines of progressively decreasing slope (Fig IV 5) Because of the speed of the early portions of this exchange reaction, the first portion of the curve can only be approximated.

The steps of the reaction have been numbered chronologically with that portion of the exchange of phosphorus which occurs between

roughly 1 and 10 hours, being Step II All that precedes this is noted as Step I Steps II and III which have rates applicable to a kinetic study are the portions of the exchange reaction to be considered in detail Based on our concept of a four-compartment system

Step I must represent the exchange of ions between the bulk solution and the chemisorbed double layer or hydration shell

Step II must represent an exchange of ions between the hydration shell and the crystal surface

Step III must involve an exchange between non-hydrated surface positions and the successive layers of intracrystalline positions.

These assignments are borne out by a kinetic analysis of the effects of ionic strength on *rates* of exchange.

In deriving a rate expression to describe these processes, it is necessary to remember that an exchange reaction is reversible It has been pointed out that isotopic exchanges follow first-order kinetics, regardless of the mechanism or of the order of the individual reactions with respect to the actual chemical constituents (20) Thus the net rate equation is

$$\frac{dx}{dt} = R\left(\frac{ay - bx}{ab}\right)$$

in which a is the number of exchangeable phosphate ions in the solid b is the number of exchangeable phosphate ions in the solution x is the number of labeled phosphate ions in the solid, y is the number of labeled phosphate ions in the solution, and R is the gross rate of exchange But since

$$y - y = x - x$$

and

$$\frac{x}{y} = \frac{a}{b}$$

in which the subscript e represents equilibrium values,

$$\frac{dx}{dt} = \frac{R}{ab}\left[(a + b)(x - x)\right]$$

This integrates into

$$\ln\left[\frac{x}{x - x}\right] = \frac{R}{ab}(a + b)t$$

or

$$\log_{10}(y - y) = Kt + \ln x$$

where K is a constant equal to

$$R\,\frac{a + b}{2.3\,ab}$$

This rate equation applies only to a single process, whereas in this instance there were at least three separate reactions occurring simultaneously If however the reactions exhibit widely different rates and therefore for practical purposes, one is in equilibrium before the next begins (i.e. $SA_{solution} = SA_{hydration\ shell}$ in the analysis of Step II and $SA_{solution} = SA_{hydration\ shell} = SA_{on\ face}$ in the analysis of Step III) it is permissible to apply the equation to the separate portions of the over-all curve

Thus a plot of the log of the activity of isotopes in solution (proportional to y) versus time, as in Figure IV 5 would give a curve comprised of at least three approximately linear portions, *provided that y is negligible with respect to y* in each case. Actually y cannot be negligible (20–30 per cent of y in Step II and approximately 10 per cent of y in Step III) but the curvature to be expected was within experimental error

Because of the uncertainties in this kinetic analysis no absolute rates can be determined for these reactions. However qualitative comparisons of rates under varying conditions can be made. For this purpose a rate term is derived from the negative of the slope of a semilogarithmic plot of the course of the reaction. Since the slope does not give a true rate, it will be referred to as the K value in the following discussion.

Variations in the ionic strength of the bulk solution can be expected to affect the rates of exchange only in the water-containing compartments. If our assignment of the reaction steps to our space model is correct, then only Steps I and II can be influenced by ionic strength. In Figure IV-6 are plotted the variations in K values for Steps II and III when the ionic strength was varied by additions of KCl to the buffer solution. The data from both Steps II and III are consistent with the Brønsted equation (10)

$$\log k = B + Z_a Z_b \sqrt{\mu}$$

where k is the specific rate constant B is a complex constant, μ is the ionic strength and $Z_a Z_b$ is the product of the ionic charge, Z of the reactants a and b Step II is markedly affected by ionic strength. Step III is independent of ionic strength a fact from which it may be concluded that the reactants are out of solution and *within* the crystal, since the product $Z_a Z_b$ is obviously not zero in this case. This conclusion is valid, regardless of the complexities involved in the evaluation of the K value Thus, even though Step I could not be analyzed, the

effects of ionic strength variations confirm the assignment of these separate exchange steps to our four-compartment model

Further confirmation of the applicability of the model was given by a study of the effect of varying the concentration of phosphate in the bulk solution Since we have postulated that the phosphate ions of the hydration shell are held by the charge asymmetry of the crystal surface, Step II—the exchange of ions between the surface and the hydration shell—should be relatively independent of variations in the phosphate content of the bulk solution. This expectation was borne out

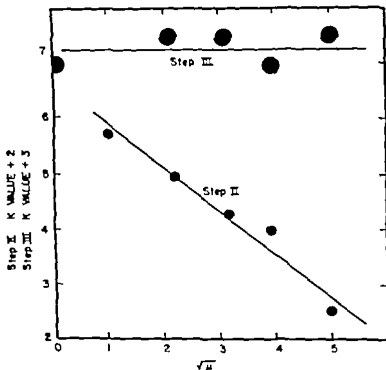


FIG. IV-6.—The effect of ionic strength on the *K*-values of Steps II and III

experimentally the *K* value for Step II was independent of both the concentration of non isotopic phosphate (from 3 to 50 γ P/ml) and the concentration of radiophosphate (from 600 to 1,200 cpm/ml) in the bulk solution.

Factors Affecting Composition of Hydration Shell

As we have seen, the rates at which radioactive phosphate is transferred from one compartment to another vary from minutes (Step I)

to days (Step III) This wide separation in rates of exchange makes possible a rough evaluation of the *size* of the reacting compartments—in this case, the amount of phosphate in the hydration shell and in the surface and the extent of intracrystalline exchange. Thus, as illustrated in Figure IV 7 Step II can be extrapolated to zero time to a value for the P^{32} in solution at the completion of Step I The reduction in solution of P^{32} given by *A* represents the dilution of radioactive label by the non-isotopic phosphate in the hydration shell only Similarly extrap-

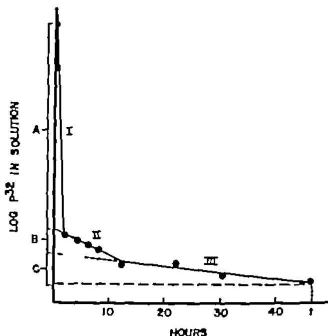


FIG IV 7 —A time-disappearance curve of radiophosphate exchange showing the extrapolation method of evaluating exchange compartment sizes (see text for full explanation)

olation of Step III to zero time gives the dilution of radioactive phosphate, *B* by the surface itself Theoretically the difference, *C* between the extrapolation value of Step III and the observed solution activity at time *t* though not at final equilibrium, should be a rough measure of the intracrystalline phosphate that has exchanged in time *t* Practically the rate of Step III is so slow that the slope of the extrapolation line is difficult to determine accurately in short term experiments. The quantities *B* and *C* then, are subject to large error In Table IV 2 are the values obtained in this way for the calcium and phosphate in the

hydration shell and surface of L-apatite Because of the uncertainties in the calculation of B and C only A will be discussed in detail. This quantity gives a measure of the amount of phosphate residing in the hydration shell by means of the usual isotope dilution equations (18 29 43)

$$A = Ri - Re$$

$$P = \frac{A \times P}{Re}$$

$$\text{Per Cent } P_{es} = \frac{P_{es}}{P_t} \times 100$$

where Ri is the initial concentration of P^{32} in solution (cpm/ml) Re is the extrapolated (Step II) equilibrium value (cpm/ml) P is the total quantity of phosphate in solution (mg. P) P is the quantity of phosphate in the hydration shell (mg P) and P_t is the total phosphate in the solid phase (mg P) All results in the experiments to be discussed subsequently will be expressed as per cent P_{es}, which in terms of our hypothetical model given in Figure IV 3 is the percentage of the total phosphate of the crystal–hydration-shell complex which resides in the hydration shell

Experimental conditions—Regardless of the conditions chosen for the exchange some time is required for the solid phase to reach a solubility equilibrium If radioactive phosphate is added to the system during the period in which the solid is dissolving the uptake of radioactivity will follow an irregular course with time (64) Any period of stirring over 18 hours was found in most cases to give solubility equilibriums. The curve of the disappearance of isotope from solution with time is then smooth and regular and the results are reproducible as in Table IV-4 column E. These data cannot be generalized, however it seems possible that, with other preparations, longer periods of pre-equilibration may be required.

Though conditions will vary with the size and pitch of the stirring blade as well as with the design any rate sufficient to keep the solid in a uniform suspension proved adequate. At very high speeds, technical difficulties are encountered particularly with the ground-glass seal, which shows a tendency to overheat and occasionally to bind. Under the conditions employed in these experiments, both the amount of exchange and the rate of the exchange were found to be independent of the rate of stirring as illustrated in Table IV-4 columns C D and E.

As discussed in chapter II the solubility of basic calcium phosphate

varies with the solid to-liquid ratio The values of per cent P_{ex} also were found to vary with the solid to-solution ratio This means that, for accurate comparisons, the solid to-solution ratio must be kept constant in any given series of experiments.

Surface area.—We should expect the magnitude of per cent P_{ex} to be a direct and linear function of the specific surface if all other variables are kept constant. The ratios of specific surface L-apatite/Kahlbaum apatite, and per cent P_{ex} L-apatite/Kahlbaum apatite, were 4.0

TABLE IV-4

COMPARISON OF SURFACE AND EXCHANGE MEASUREMENTS ON TWO APATITE PREPARATIONS

Apatite (Ca/P)	Surface Area	Stirring Rate (r p m)	Step II K Values ($Hr^{-1} \times 10^3$)	Hydration-Shell Phosphate (Per Cent P_{ex})
A	B	C	D	E
Kahlbaum (1.53)	16.8	200	1.26	0.92
		200	1.54	.88
		300	1.57	.94
		300	1.66	0.81
				0.89±0.04
L-apatite (1.66)	67.8	200	4.40	3.37
		200	4.65	3.49
		1 600	4.22	3.20
		1 600	4.65	3.73
				3.45±0.12

and 3.9 respectively indicating a close correspondence between surface area and per cent P_{ex}.

Effect of temperature.—Most of the studies described were arbitrarily carried out at body temperature (37°) Although the actual temperature at which the exchange occurs is not important per se, a valid comparison of a series of exchanges can be made only when they are conducted at one temperature From the data in Figure IV-8 it can be seen that increasing temperature markedly decreases the amount of hydration-shell phosphate, per cent P_{ex}. This effect is difficult to interpret with present limited knowledge.

Effect of phosphate concentration.—Because of the analytical necessity of distributing the isotope more or less equally among the compartments, it is impractical to study the exchange process at very high or

very low phosphate concentrations. However as illustrated in Table IV 5, within the range of phosphate concentration which might be encountered in this study the amount of phosphate in the hydration shell was independent of the concentrations of phosphate ion in the bulk solution. Over wider limits of variation one might expect alterations in the charge on the crystal surfaces and as a result, corresponding alterations in per cent P_{ex}.

Effect of calcium concentration —Experimental conditions permitted a much wider variation in calcium concentration. As discussed in chapter iii, the hydroxy apatite crystal shows variations in calcium-phosphate ratios which are best explained as resulting from replacement of Ca^{++} by $2H_3O^+$ Whether these substitutions occur in the crystal in the surface or in the hydration shell or in all three is at present somewhat

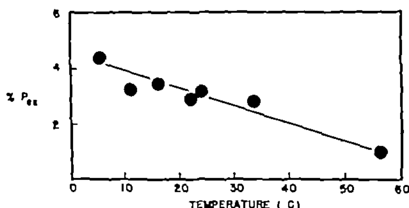


FIG. IV-8.—The effect of temperature on per cent P_{ex}

TABLE IV-5*

CONSTANCY OF PER CENT P_{ex} WITH VARYING PHOSPHATE CONCENTRATIONS IN SOLUTION

Phosphate Concentration (mM/l)	Per Cent P_{ex}
0 06	4 4
0 07	4 7
0 27	4 6
0 30	4 3
0 33	4 5
0 47	4 3
1 7	5 0

* μ = 0 165; pH = 7 2; T = 37° C.

uncertain Excessive substitutions at the surface or within the crystal can be expected to alter the surface-charge asymmetry and thus the composition of the hydration shell The charge asymmetry would be related to the $[Ca^{++}]/[H_3O^+]$ of the bulk solution provided that other competing cations are absent Evidence for such a relationship was obtained as shown in Figure IV 9 where the $[Ca^{++}]/[H_3O^+]$ had been varied by additions of calcium, while pH μ etc. were kept constant The large increase in per cent P_{ex} with increasing $[Ca^{++}]/[H_3O^+]$ could

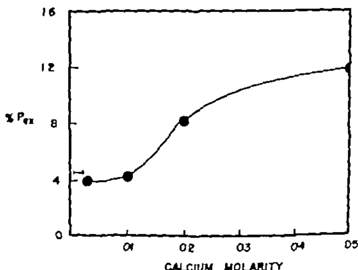


FIG IV-9 —The effect of excess calcium ion concentration in solution at constant pH on the per cent P_{ex} The symbol ↦ designates the data obtained with varying phosphate concentrations plotted on a comparable scale (cf Table IV-5)

have resulted from either a change in the charge asymmetry of the crystals or a change in crystal size. This latter possibility was eliminated by surface-area determinations of the crystals made before and after the equilibration. The two values were in agreement, 68 M^2/gm indicating that the change in per cent P_{ex} occurred from changes in the crystals charge asymmetry only

Relation between Rates of Exchange and Per Cent P_{ex}

As noted above a number of variables—temperature, μ solid-solution ratio pH and calcium concentration or $[Ca^{++}]/[H_3O^+]$—affect the magnitude of per cent P_{ex} the phosphate in the hydration shell. It is of interest that with increasing values of per cent P_{ex} decreasing rates of exchange (decreasing values of K for Step II) were obtained, with

only one exception when small amounts of calcium were added to the buffer solution K increased with *no change* in per cent P_{ex}. This general inverse relation between *rate* of surface exchange and the *amount* of hydration-shell phosphate is of prime importance in elucidating the mechanism of the Step II exchange process.

MECHANISMS OF ISOIONIC EXCHANGE

The data obtained in these studies along with other information are sufficient to permit a partial analysis of the actual reaction mechanics of the over-all exchange process.

Even though Step I was so rapid that it could not be subjected to a kinetic study the rate itself indicates the most likely mechanism to be a self-diffusion process. The simple diffusion of ions between the hydration shell and the bulk solution would account for the rapid rate and is consistent with the entry of simple solute ions, such as K^+ or Cl^- as discussed subsequently.

Step II involves a less obvious mechanism and because these experiments were carried out at equilibrium there is no justification a priori for assuming the rate-determining step to be the transfer of labeled ions from the hydration shell to the surface. Just as likely the transfer of non-isotopic ions from solid surface to hydration shell might be rate-determining. Indeed the data are best explained by assuming the rate-determining step to be escape or self-diffusion of phosphate ions from the crystal surface to the hydration shell—a mechanism consistent with the facts. In terms of our model, the rate of escape of an ion into the hydration shell should decrease with an increase in the opposing electrical potential in the double layer. Thus the greater the electropositivity of the surface, the greater the amount of bound, polarizable phosphate ions required in the hydration shell to neutralize the charge asymmetry. Both these changes would oppose the thermal escape of the surface phosphate ion. We have just seen that there is a decreasing rate of exchange in Step II with increasing values of per cent P_{ex} irrespective of the variable inducing the change—μ $[Ca^{++}]/[H_3O^+]$ or temperature. Increasing temperature, by itself would be expected to increase the rate of exchange. However because per cent P_{ex} increases with increasing temperature, the net effect is a reduction in rate. Apparently the electric potential barrier is the most important variable affecting the rate of exchange. The effect of ionic strength, too is easily explained in these terms. With increasing ionic strength, per cent P_{ex} increases, producing an increased potential barrier and thus a

reduced rate of escape of surface phosphate ions. Were it necessary to interpret the effect of ionic strength in terms of a conventional collision mechanism both the sign and the slope (−0 78) of the curve given by the Brønsted plot (Fig. IV-6) would be difficult to relate to the system under study

If the rate of isotope uptake is indeed governed by the escape of surface ions, the assignment of the observed reaction steps to the proper compartment in our crystal-solution model can be made with some assurance. Thus Step III evincing a rate independent of ionic strength, must represent a reaction originating within the crystal. Step II dependent upon ionic strength must represent a reaction originating either on the surface or in the hydration layer Since Step I is a reaction much faster than Step II Step II remains, by elimination, as the escape of surface ions to the hydration layer

Step III exchange *within* the crystal has been described in earlier studies and has, under various conditions, been variously termed "thermal aging' (29) and recrystallization (43) Certainly the process here described does not involve a dissolution and redeposition of crystals, because the rate is independent of the ionic strength of the solution. The experimental period in these studies involved only a minute fraction of the lifetime of the well-aged crystal. For these reasons, *intracrystalline exchange* is the preferred term. Intracrystalline exchange is strongly temperature-dependent, increasing markedly in rate with small temperature increases (14 43) Under constant conditions the rate is not constant but appears to decline steadily over a period of 44 days (63) Step III thus involves a series of declining rates as the labeled ions diffuse into successively deeper layers of the crystals. While it is possible that certain ions may be able to diffuse" throughout the lattice of certain crystals, in the present instance, where the lattice is quite dense and where the diffusing ion—phosphate—is large and multicharged it seems more probable that ion migration is mediated through the presence of unoccupied lattice positions or defects known to be present in hydroxy apatite crystals (cf. chap. iii) This view is supported by the observation that high-temperature ashing markedly reduced the slow phase of isotope uptake by fresh bone, termed at that time "recrystallization ' (43) Such heat treatment would be expected to render the crystals more perfect, with fewer defects and vacant positions, as suggested by improved X ray diffraction patterns (44) The participation of 'holes which move as the result of thermal vibration

of adjacent ions is also consistent with the ever decreasing rate of reaction as the migrating ion penetrates deeper layers of the crystals because the concentration of defects is said to be minimal at the crystal center (24) With larger aged crystals, the reaction rate falls so rapidly that one must conclude that only a few "molecular layers" can take part in an observational period of a few weeks (65)

In cases where hydroxy apatite crystals are being formed or are growing experience has shown that there are sequential changes in crystal morphology (5 62) Such changes suggest the occurrence of a process of recrystallization in the classic sense a dissolution and redeposition of crystals. There is some evidence that this conventional type of recrystallization also takes place in areas of new bone formation (67)

Grimley (24) has attempted a mathematical analysis of exchange in an ionic crystal in contact with aqueous solutions. He limits his analysis to a simple binary salt crystal (AgBr) which exhibits normal solubility or K_{sp}, and is in contact with a saturated solution containing no supporting electrolyte. He has difficulty in relating his analysis to the practical case—even the AgBr system It is doubtful therefore, whether his conclusions or better his predictions can be transferred to the hydroxy apatite–aqueous system in more than a semiqualitative way His findings are so interesting however that they will be listed as a guide for what to expect in the way of future results

a) All crystals contain a large number of vacant positions and interstitial ions (Schottky and Frenkel defects)

b) Only at the isoelectric point are the cationic and anionic defects equal in number

c) Above the isoelectric point (which is equivalent to high Ca/P ratios in solution) the crystals are positively charged, and anionic exchange *within* the crystal is facilitated cationic exchange repressed

d) Below the isoelectric point (low Ca/P ratios in solution) the crystals are negatively charged and cationic self-diffusion is facilitated anionic exchange repressed.

e) Very thin (<500 A) platelike crystals have an aberrantly high number of defects and their exchange behavior will be very sensitive to changes in solution composition.

As seen below some of Grimley s predictions have already been substantiated by recent studies of calcium exchange with Ca^{45} as a tracer

CALCIUM EXCHANGE IN MODEL SYSTEMS

In contrast to our understanding of phosphate exchange, which, if somewhat provisional is detailed our knowledge of the kinetics of calcium exchange is very limited There is an indication that the over-all process is very similar to that of phosphate in exhibiting a three-step

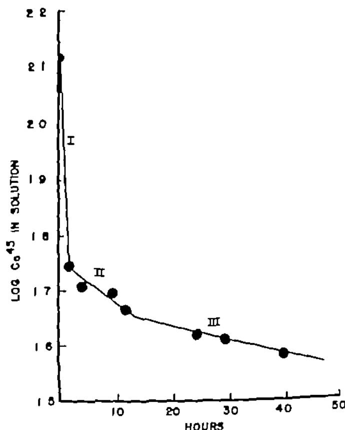


FIG IV 10 — The stepwise disappearance of Ca^{45} from solution. Taken from Weikel (63)

time curve, as in Figure IV 10 (63) but the effects of temperature, p, etc have not been examined under equilibrium conditions.

As with Step III of the phosphate exchange, Step III occurs at an ever decreasing rate with passing time (Fig IV 11) In addition, at 44 days, over 17 per cent of the total crystal calcium has exchanged, a quantity too large to be attributed to the crystal surface (65) This is strong presumptive evidence that Step III again represents intracrystalline exchange If cationic exchange were truly analogous to phos-

phate exchange the rate-determining reaction in Step II would be the thermal escape of unlabeled Ca^{40} from the crystal surface to the hydration shell *irrespective of the exchanging labeled cation* That this is true is indicated by the observation that the rate of Step II or the K value is the same whether the exchanging cation is Sr^{89} or Ca^{45} (8) Finally Step I is an extremely fast exchange and must involve the outermost ions, the calcium ions located in the hydration shell. From these analogies we may presume that the three-step exchange of Ca^{45}, like that of

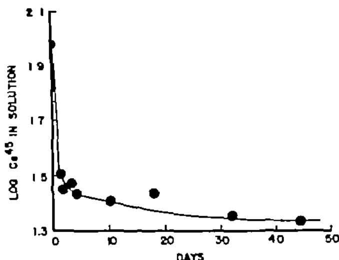


FIG. IV 11 —A long term calcium exchange, showing a continued but progressively slowed removal of Ca^{45} from solution by the solid phase In this experiment eight identical flasks containing apatite and Ca^{45} were rotated at a constant temperature of 37° C. and were sampled serially for radioactivity assay and calcium concentration of the liquid.

$P^{32}O_4$, represents the sequential distribution of Ca^{45} among the four compartments of our crystal-solution model Presumably also the extrapolation procedure, described previously for estimating the amounts of exchangeable ion in the hydration shell and surface is as applicable for Ca^{45} as it is for $P^{32}O_4$.

Nonetheless, further study is badly needed. The presence of carrier in all but the very expensive carrier free Ca^{45} preparations has rendered attempts at a kinetic approach (under equilibrium conditions) almost impossible Despite this difficulty and despite our lack of knowledge of the isoelectric point of the hydroxy apatite crystal, there is some evidence that points *c* and *e* of Grimley's predictions given earlier are

operative For example, Dallemagne and associates have demonstrated (13 14) that the slow exchange process in hydroxy apatite crystals (presumed here to be intracrystalline exchange) is markedly depressed by increasing the Ca/P ratio of the equilibration fluid It was shown further by Fabry that the Ca/P ratio of preformed crystals is easily altered by changes in the Ca/P ratio of the solution (17)

The problems imposed by the responsiveness of the crystals to the conditions in their fluid environment render it difficult to evaluate and compare different studies of ion exchange in hydroxy apatite preparations and impossible to compare them quantitatively with bone mineral. The amounts of exchangeable calcium and phosphate are determined not only by the solid phase but also by the conditions under which the exchange measurements are made μ, pH, Ca/P ratio, solid-to-solution ratio Ca^{++}/H_3O^+ ratio and temperature.

There are some results which at first glance, might argue against this rather pessimistic analysis of the literature. Olsen (48) for example, reports that apatites of different size and composition in different equilibrating solutions give the same exchange value as measured by $P^{32}O_4$ when expressed as 0 238 mg P/M² surface area (equivalent to $3PO_4^{\equiv}$/surface unit cell) How is it that this author is not pestered by the complexity of the system under study? An analysis of his data reveals the following critical points (*a*) he studied only three preparations of apatite all of which were phosphate-rich apatites (low Ca/P ratios) (*b*) all were studied at low solid-solution ratios at solubility equilibrium in solutions of low Ca/P ratio From the preceding discussion it follows that under these circumstances the surface was more or less saturated with phosphate and that intracrystalline exchange of $P^{32}O_4$ was minimized i.e. an apparent exchange "equilibrium" was quickly reached. Thus his measurement of "surface phosphate" may be presumed to be the sum of Steps I and II and represents the sum of the phosphate residing in the hydration shell (P_{sh}) and in the crystal surface. Measuring P_{sh} only Weikel found he could vary the quantity of phosphate in the hydration shell from less than 2 to more than 12 per cent of the total phosphate in the same apatite preparations, or less than 0 6 to over 3 6 molecules of phosphate per surface unit cell (65)

MAGNITUDE OF EXCHANGE IN BONE MINERAL

Because of the many variables which affect the magnitude of the exchange, it is difficult to evaluate the published reports on exchange in bone in vitro Even if one were to use carefully defined conditions and

the most recent techniques to study exchange in bone preparations, it is doubtful whether the results would have quantitative meaning How should the bone mineral be prepared for study? All manipulative techniques alter the conditions that obtain in vivo Any sample of sufficient size to be conveniently studied will represent an average of wide variations in exchange at the histological level Even so the range in reported exchange values is interesting With fresh bone examined under a variety of conditions total exchange values vary from 5 to 50 per cent for phosphate and from 5 to 27 per cent for calcium (11 13 15 18 43 61) In general, low temperature ashing increases the extent of exchange of bone samples, while high temperature ashing nearly abolishes it (40 43) Areas of new growth exhibit much more active exchange than does matured compacta both in vivo and in vitro (2, 4) These observations are undoubtedly related to the water content, the presence of organic material and the crystal size. Low water content and the presence of protein fibers prevent the access of the labeled ion to the crystals. Low temperature ashing removes the organic material and permits better penetration of water and of isotope. High temperature ashing, on the other hand, invokes crystal growth with concomitant loss in surface area and reduced intracrystalline defects which mediate intracrystalline exchange. In areas of new growth the factors promoting extensive exchange are smaller crystals, more lattice defects, and greater hydration. The maximum of 50 per cent phosphate exchange listed above was obtained on such crystals scraped from newly forming periosteal surface.

It is doubtful then, whether much useful information will be derived from further studies of the exchange of powdered bone or bone ash in vitro The main benefit to be derived from such studies is, of course, a partial bridging of the gap between the purely inorganic, physicochemical model system and the complex system in vivo A more promising approach would be to apply quantitative radioautography to thin, histological sections of intact bone tissue which have been exposed to radioactive buffers.

Heteroionic Exchange

Substitution of lattice positions by ions foreign to the hydroxy apatite lattice has been discussed in chapter iii. The number of such substitutions is rather limited but, at the surfaces of the crystals where space-charge requirements are less restrictive, the opportunities for substitutions are much greater In the hydration shell, with its concen-

tration of bound multicharged polarized anions and hydrated cations, a wide variety of ion substitutions is possible. In fact, such ion substitutions—*heteroionic exchange*—permit hydroxy apatite crystals to mirror the composition of fluids in which they are placed. The complex process of heteroionic exchange is the primary determinant of the composition of bone mineral and warrants a detailed examination.

The mechanisms by which the hydroxy apatite crystals mirror the composition of their fluid environment are those described above, elucidated by exchange studies with $P^{32}O_4^{\equiv}$ diffusion into the hydration shell and exchange with ions in the bound layer, in the surface, and in the crystal interior. Since there is considerable uncertainty as to the precise ionic properties which are responsible for an affinity for each compartment of the crystal-water system, each ion must be tested individually for its differential distribution. There are, at present, two general methods by which this differential distribution can be investigated.

Ions which are not selectively concentrated by hydroxy apatite can be studied only by means of a centrifugal isolation of the hydrated crystals free from contaminating, bulk solution. The uptake of ion by the solid phase can then be determined directly and related to the concentration of the ion in the solution phase at equilibrium. A typical study of this type involving sodium and potassium ions (56) will be described in detail.

Ions which are concentrated by hydroxy apatite crystals are more easily and definitively studied by a kinetic analysis of the disappearance of the ion from a solution containing hydroxy apatite crystals. The procedure is analogous to that described above using $P^{32}O_4$ and Ca^{45}. A requirement of this kinetic approach is a strict maintenance of equilibrium conditions with respect to $[Ca^{++}]$, $[PO_4^{\equiv}]$, μ, and pH. Thus only minute quantities of the ion under investigation can be added to the equilibration solution. The method is applicable, therefore, only for those ions for which high specific activity radioisotopes or highly sensitive analytical procedures are available. Where applicable, the kinetic approach gives the most detailed information concerning the distribution of an ion in our four-compartment model.

From the information derived from these two experimental approaches it is possible to differentiate the four main classes of ion interactions with hydroxy apatite crystals.

I. Ions which diffuse into the hydration shell but do not concentrate there. A steady state is attained in a few hours or less, the system is

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From the information derived from these two experimental approaches, it is possible to differentiate the four main classes of ion interactions with hydroxy apatite crystals

I. Ions which diffuse into the hydration shell but do not concentrate there A steady state is attained in a few hours or less, the system is

readily reversible and the concentration of the ion in the hydrated solid is directly proportional to its concentration in the bulk solution

II Ions which enter the hydration shell and participate in the neutralization of surface-charge asymmetry A steady state is attained in a few hours, the system is readily reversible and the concentration of the ion in the hydrated solid is a function of its concentration in solution relative to the concentration of the ion being displaced calcium phosphate or hydroxyl

III Ions which enter the hydration shell and replace ions in the crystal surfaces. These ions may or may not also participate in the neutralization of surface-charge asymmetry Again a steady state is attained in a few hours, the system is readily reversible, and the concentration of the ion in the hydrated solid is a function of its concentration in solution relative to the concentration of the ion being displaced

IV Ions which can displace lattice ions both in the surface and in the crystal interior A steady state cannot be demonstrated over periods of months the system appears to be irreversible, and the concentration of the ion in the solid phase is a function of its concentration relative to the concentration of the ion being displaced and the duration of the equilibration.

It is not always easy to differentiate between Class II and Class III interactions, and a variety of procedures has been employed to clarify gross data on ion binding by hydroxy apatite. These procedures will be described where employed.

Lacking an experimental study in vitro one can sometimes deduce the probable distribution of an ion by comparing its physiological behavior in vivo with that of an ion which has been adequately investigated.

In general, it has been found that monovalent ions (K^+ Na^+ Cl^- F^-) diffuse into the hydration shell but do not concentrate there Multivalent anions and cations which are highly hydrated and/or polarizable (Mg^{++} Sr^{++}, Ra^{++} UO_2^{++}, $CO_3^=$ [citrate$^=$?]) tend to concentrate in the hydration shell becoming part of the bound ion complex. Some of these (Sr^{++} Ra^{++} $CO_3^=$) enter the crystal surface itself In addition, two monovalent ions (Na^+ F^-) are known to enter the surface Some few ions (Sr^{++} Ra^{++} F^-) can even enter the crystal interior and participate in intracrystalline exchange. The ultimate distribution of some of these ions (Mg^{++} UO_2^{++} and citrate$^=$) is not completely known they seem to be surface limited, but whether these substitutions involve the

bound ion layer and also the crystal surface is not certain In all cases these specific interactions involve a displacement of at least one of the normal lattice ions cations displace calcium, multivalent anions displace phosphate, and fluoride displaces hydroxyl.

SODIUM AND POTASSIUM BINDING BY HYDROXY APATITE CRYSTALS

The well-characterized L-apatite employed throughout these studies contained less than 0 01 per cent of sodium. Equilibrations were carried out in the apparatus described previously (Fig IV-4) The solutions contained sodium and potassium chlorides in varying proportions, while the sums of their concentrations were kept constant to maintain a constant ionic strength. The solutions were weakly buffered with diethylbarbituric acid at pH 7 4 Two grams of L-apatite were equilibrated with 1 liter of solution for 18 hours, which was shown to be adequate to reach a solubility equilibrium. The sodium content of the solid became constant within 2 hours.

At the end of the equilibration period, approximately 30 ml of the solution were withdrawn by suction through a very fine sintered glass filter for calcium and phosphate analyses. The remainder of the suspension was filtered and the crystals with their hydration shells, but free from contaminating bulk solution were isolated by high-speed centrifugation (46) The solid was then dried and analyzed for sodium and potassium content by flame photometry The results (56) are given in Figure IV 12.

Under conditions where temperature, pH, and ionic strength are all constant the surface-charge asymmetry of the crystal and the volume of the hydration layer may be assumed to remain constant and therefore the amount of cation taken up by the solid due only to *diffusion* into the hydration layer is given by the relation

$$f_{M} [M'] = k f_{M} [M] \quad \text{(1)}$$

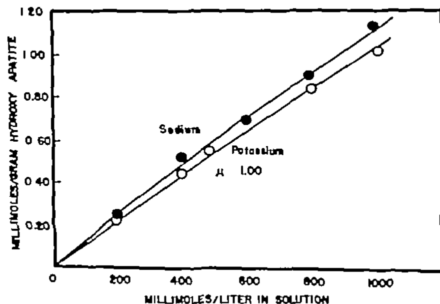
with subscripts under the bracketed terms: (hydration shell) and (solution)

or

$$[M'] = k \frac{f_M}{f_{M'}} [M] \quad \text{(2)}$$

where M is the ion under study f designates the activity coefficient, k is the proportionality constant, and concentrations are indicated by brackets. This relation states that the amount of cation in the solid phase is directly proportional to the concentration of the cation in solution, *provided that the cation does not exchange for other cations in the crystal surface or participate in the neutralization of the surface-charge*

a



b

FIG. IV 12 *a* and *b*—Uptake of sodium and potassium by hydrated hydroxy apatite.

asymmetry The data given in Figure IV 12 *a* show that equation (2) describes the binding of potassium very well At $\mu = 0.16$ the curve is linear within experimental error at $\mu = 1.00$ Figure IV 12 *b* there is a slight curvature which may be ascribed to small changes in activity coefficients as the mole fractions of potassium $K^+/(Na^+ + K^+)$ vary from 0 to 1

Since, under equilibrium conditions, the chemical activities in both solid and solution phases are equal, it may be assumed that the proportionality constant in equation (1) is equal to unity With this assumption the activity coefficient, $f_{M'}$ of potassium in the hydration shell can be calculated thus

$$f_M = k f_M \frac{[M]}{[M']}$$

where f_M at $\mu = 0.16$ is 0 72 (from Table I 1) and the value $f_{M'} =$ 0.55 Thus the activity of potassium in the hydration shell is considerably lower than that in the bulk solution It seems only reasonable that ion ion interaction and ion-solvent interaction would be greater in a charge-oriented hydration layer than in an ordinary solution particularly since there exists a high concentration of multicharged ions in the bound layer

Equation (2) does not predict accurately the uptake of sodium ion by the solid This is not surprising. It is a long-established fact that bone mineral when boiled in non-aqueous alkali, takes up great quantities of sodium but not potassium For this reason, the classical ashing procedures have always employed KOH. Apparently the sodium ion can react with the crystal even in non-aqueous medium. In aqueous medium it appears that the calcium ion is displaced from the solid by the entering sodium ions. Typical data, suggesting that the sodium in the solid is a function of its concentration relative to calcium in solution are given in Figure IV 13 At the moment it is not settled whether sodium is displacing calcium ions in the bound ion layer of the hydration shell or in the crystal surface itself or both. Were the reaction in the lattice surface, we should expect 1 sodium ion (of radius 0 95 A) to displace 1 calcium ion (of radius 0 99 A) Were sodium functioning in the dilution of charge asymmetry in the hydration shell we should expect that 2 monovalent sodium ions would be needed to replace 1 divalent calcium ion. The sodium ion is less hydrated and polarizable than is the calcium ion. A partial answer to the location of sodium exchange can be obtained by a mathematical analysis of the data.

The sodium ions entering the crystal surface or the bound double layer by exchange, $[Na]_{ex}$ may be represented by the total sodium content minus that sodium located in the hydration shell as a result of diffusion only

$$[Na]_{ex} = [Na]_{total} - [Na]_{diff}$$

While $[Na]_{total}$ is observed $[Na]_{diff}$ must be estimated If it is assumed that $[Na]_{diff} = [K]_{diff}$ under equivalent conditions, since the fact that $[K]_{diff} = [K]_{total}$ has been established then

$$[Na]_{ex} = [Na]_{total} - [K]_{total}$$

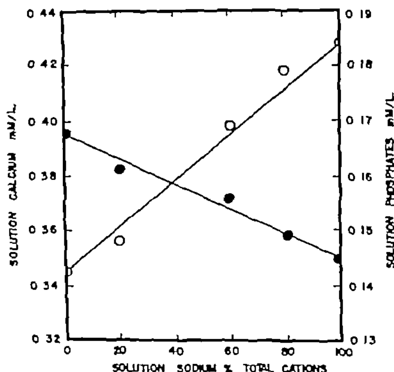


FIG IV 13 —The displacement of calcium ions from hydroxy apatite by sodium ions at constant ionic strength $\mu = 1.00$· ○ = calcium ● = total phosphates.

and the sodium uptake resulting from *exchange* can be obtained by subtracting the potassium uptake curve from the observed total sodium uptake curve (cf Fig IV 12) (It is obvious that most of the sodium entered the hydrated solid by simple diffusion.) From the law of mass action, it follows that

$$[Ca]_{ex} + [Na]_{solution} = [Na]_{ex} + [Ca]_{solution}$$

or

$$[\mathrm{Na}]_{ex} = k\,[\mathrm{Ca}]_{ex}\left(\frac{a_{N}}{a_{\mathrm{Ca}}}\right)_{solution} \quad (3)$$

where $[\mathrm{Ca}]_{e}$ represents the concentration of calcium ions in the crystal surface and/or the hydration-shell double layer. If two sodium ions replace a single calcium ion,

$$[\mathrm{Ca}]_{ex} + 2\,[\mathrm{Na}]_{solution} = [\mathrm{Na_2}]_{ex} + [\mathrm{Ca}]_{solution}$$

or

$$[\mathrm{Na_2}]_{ex} = k\,[\mathrm{Ca}]_{ex}\left(\frac{a^2_{\mathrm{Na}}}{a_{\mathrm{Ca}}}\right)_{solution} \quad (4)$$

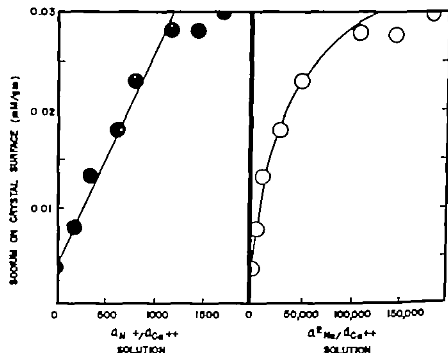


FIG. IV 14.—Indication of a mole-for mole ionic competition between sodium and calcium with hydrated hydroxy apatite crystals $\mu = 0.162$.

In either case, linear relations between $[\mathrm{Na}]_{ex}$ and $(a_{\mathrm{Na}}/a_{\mathrm{Ca}})_{solution}$ or $(a^2_{\mathrm{Na}}/a_{\mathrm{Ca}})$ can be expected only at low levels of sodium impregnation where the term $[\mathrm{Ca}]_{ex}$ remains essentially constant. When the data are plotted according to equations (3) and (4) as in Figure IV 14 a mole-for mole exchange is indicated. This suggests that the major exchange reaction is a surface exchange. The lack of charge equivalence would be expected to reduce the positive charge on the crystal's surface, resulting

in a loss of neutralizing anions in the hydration shell It has indeed been shown (65) that sodium impregnation sharply reduces the con centration of phosphate in the hydration shell per cent P_{x}, as meas ured by exchange techniques.

There is some indication that not all the surface calcium positions are available for sodium exchange. Crystals suspended in solutions where the molar ratio Na/Ca = 10 000 take up only 0 1 mM/gm of $[Na]_{x}$. This particular apatite has been shown (Table IV 2) to contain 0 2 mM Ca/gm apatite in the hydration shell and 0 15 mM Ca/gm apatite in the crystal surface. At physiological concentrations of sodium, only about 25 per cent of the surface calcium ions are displaced by sodium ions.[1] This represents a relatively weak affinity for apatite The presence of sodium in bone must be due mainly to its high con centration in physiological fluids.

The question arises as to whether the mechanisms involved in so dium uptake by a synthetic apatite in vitro can be extrapolated to the physiological state of bone mineral in vivo A final answer cannot be given but the sodium content of bone can be reasonably explained in terms of the hydration-shell diffusion and crystal surface exchange. The crystals of bone are smaller than the crystals of apatite used in these studies (68 M^2/gm) and therefore present a greater surface area probably approaching 300 M^2 as discussed earlier On the other hand bone crystals are not fully hydrated averaging only about 0 4 gm H_2O/gm apatite (46) A rough estimate of the expected sodium content of bone can thus be calculated from the results of our model system

Bone crystal surface exchange = 0 04 mM/gm

$$\times \frac{300\ M^2/\text{gm bone ash}}{68\ M^2/\text{gm apatite}} = 0\ 18\ \text{mM/gm}$$

Bone hydration shell = 0 16 mM/gm

$$\times \frac{0\ 4\ \text{gm}\ H_2O/\text{gm bone ash}}{0.8\ \text{gm}\ H_2O/\text{gm apatite}} = 0\ 08\ \text{mM/gm}$$

Total predicted Na content in bone ash = 0.26 mM/gm

Actual analyses of bone range from 0.25 to 0.30 mM/gm ash in re markable agreement with the values predicted from the model system

1 This figure is based on experiments in vitro The $[Ca^{++}]$ was only one-tenth that of serum Mg^{++} was not present etc. For these reasons, such extrapolations are very approximate.

OTHER CATIONS

If our present conception of the crystal-surface–hydration-shell complex is correct, we may expect some day to be able to classify *all* inorganic cations and anions with respect to their interaction with an aqueous–hydroxy apatite crystal system. We may expect that the degree to which an ion concentrates at the crystal-solution interface will depend upon its valence its hydration its polarizability and its size. Unfortunately there are insufficient data at hand from which to draw such sweeping generalizations at present.

A number of cations besides sodium some physiological, some unphysiological, are found to concentrate in bone in vivo Of these, most appear to enter the mineral phase by the mechanisms elucidated in the radiophosphate and sodium studies described above.

Magnesium

Of the total body content of magnesium nearly 60 per cent resides in the skeleton (7) This skeletal depot is quite dynamic, showing gain or loss depending on the dietary level of magnesium Despite the physiological importance of this ion, no study of its interaction with hydroxy apatite crystals has yet been reported. The physiological behavior of skeletal magnesium indicates that it is a surface limited ion (hydration shell and/or surface) incapable of penetration into the lattice interior (7) This is essentially an argument by analogy the magnesium of bone behaves physiologically like the sodium or carbonate of bone. Sodium and carbonate have been studied in model systems in vitro and appear to be surface-limited. On purely crystallographic grounds, Hendricks has postulated that magnesium adsorbs" on the crystal surface as $MgOH^+$ (25) Dallemagne and Fabry (14) reported that most of the magnesium content of low temperature ashed (400°–600° C) bone can be removed by leaching with water

Taken *in toto* this fragmentary evidence suggests that magnesium displaces calcium in the hydration shell and/or surface on a mole-for mole basis (7) Poor methodology and the lack of a suitable isotope of magnesium have hampered the development of our knowledge concerning this important cation

Strontium

Interest in the strontium cation was stimulated by reports that, in conjunction with dietary calcium supplements, it was a useful adjuvant in the therapy of osteoporosis (55) Lately interest in skeletally depos-

tied strontium has increased greatly but for less humane reasons. Because long-lived radiostrontium is one of the principal fission products which persist after an atomic explosion, this ion holds a central position in the arena of argument concerning the radioactive hazard to the general population resulting from competitive Russian and American nuclear weapons testing programs. It is not surprising therefore that a rash of government-sponsored studies on the metabolism of strontium in animals has recently appeared. Strangely the *mechanism* of strontium deposition in bone has been almost neglected. This is now being remedied. Unpublished kinetic analyses of the disappearance of Sr^{++} from solutions of a model aqueous apatite system (8) have clearly shown that radiostrontium enters the crystals in the same way as does radiocalcium itself. The time course of the exchange of labeled strontium for crystal calcium follows a three-step curve, and the rates and extent of the three processes are approximately equal to those observed with Ca^{++}. This means that Sr^{++} has the properties required for participation in the neutralization of the surface-charge asymmetry as well as surface and intracrystalline exchange. It also suggests that strontium has nearly the same affinity for apatite as does calcium. Earlier evidence (33 34) and recent tissue-culture studies (51) are consistent with these general conclusions. The apparent discrimination against strontium in vivo (Sr/Ca ratios in bone mineral are from one-half to one-fifth the Sr/Ca of the diet [1 12]) must be ascribed primarily to gut (12) and kidney discrimination.

Radium

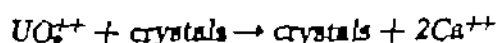
The atomic age has given great growth impetus to a new branch of biological science—radiation biology. Besides a fundamental interest in learning just how radiation affects living processes, the radiation biologist also has a somber yet practical reason for his studies. There is an urgent need for new knowledge of radiation effects as long as the possibility of nuclear warfare persists in the world. One crucial problem in this field involves the extrapolation to the human of data obtained on experimental animals. The extrapolation is usually based on the largest and best-documented group of human cases of radiation injury available—people who from their occupation (radium dial painting) or their doctor (radium water as a tonic) acquired a "body burden" of radium (32). One of the unique aspects of these cases was the extremely slow excretion of the skeletally deposited radium.

A comprehensive study of the kinetics of the exchange of radium for

calcium in the aqueous–hydroxy apatite model system has been made (42) Like strontium radium enters the hydration shell and the crystal surface and can also participate in intracrystalline exchange. After short exposure periods, the radium can be driven off the crystals by the addition of a competitive ion such as calcium to the solution. Sodium ion because of its lower affinity for apatite, is without measurable competitive influence on the radium exchange.

Uranyl Ion

Like strontium and radium, uranium owes its importance to the advent of the atomic age A primary nuclear fuel uranium, both normal and isotopically enriched, is being processed in large quantities by large numbers of people and consequently has become a potential industrial exposure hazard Wartime studies (60) had established that only hexavalent uranium, UO_2^{++} is of practical physiological importance, since all other valence forms either are not absorbed or are oxidized to UO_2^{++} in vivo The uranyl ion, which is retained in the body is found almost exclusively in the bone mineral (there is a temporary retention in the kidney) from which it is mobilized only slowly The underlying mechanisms in this skeletal deposition are similar to those of other foreign cations. Studies in vitro of UO_2^{++} fixation were made before current concepts of the crystal-surface–solution interface and current experimental techniques had been developed. We do know that the uranyl ion penetrates the hydration shell and exchanges for calcium on a molar 1 for 2 basis

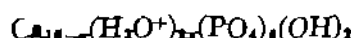


$$UO_2^{++} + \text{crystals} \rightarrow \text{crystals} + 2Ca^{++}$$

We do not know to what extent this exchange occurs in the bound-ion layer and/or the crystal surface. We might expect from its large ionic volume and polarizability that the uranyl ion would be more effective in neutralizing charge asymmetry than in exchanging for surface sites. However uranyl ion fixation is accompanied by a loss in exchangeable phosphate, which may indicate some surface exchange. This should be reinvestigated. The fact that UO_2^{++} exchange is readily reversible in vitro demonstrates that the exchange is surface-limited.

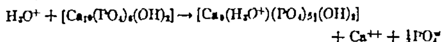
Hydronium

In earlier discussions of the crystallography of hydroxy apatite (chap iii) it was necessary to resort to the general formulation,



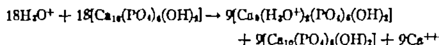
$$Ca_{10-x}(H_3O^+)_{2x}(PO_4)_6(OH)_2$$

to explain the variable stoichiometry encountered in different samples of this mineral. Inherent in this formulation is a presumed substitution of 2 moles of hydronium ion for 1 of calcium. Yet the end product of such a presumed substitution tells us nothing about the mechanism or location of the substitution. For example let us assume that H_3O^+ substitutes for lattice Ca^{++} not on a charge-equivalent basis but mole for mole, as does sodium ion, thus



$$H_3O^+ + [Ca_{10}(PO_4)_6(OH)_2] \rightarrow [Ca_9(H_3O^+)(PO_4)_{5\frac{2}{3}}(OH)_2] + Ca^{++} + \tfrac{1}{3}PO_4^{\equiv}$$

where the loss of crystal charge in substituting monovalent ions for divalent ions causes a loss of surface bound anions, as was observed in sodium exchange. By repeating this process over and over and recombining the ions released from the solid phase we obtain



$$18H_3O^+ + 18[Ca_{10}(PO_4)_6(OH)_2] \rightarrow 9[Ca_8(H_3O^+)_2(PO_4)_6(OH)_2] + 9[Ca_{10}(PO_4)_6(OH)_2] + 9Ca^{++}$$

Thus the resulting products of a mole-for mole exchange process would appear, on analysis, as a charge-equivalent exchange, $2H_3O^+$ for $1Ca^{++}$

Actually there exist some fragmentary indications that the underlying exchange is indeed non-equivalent mole for mole. Hodge in collecting solubility data from the literature observed as had Shear and Kramer (54) an empirical relation between $[H_3O^+]$ expressed as pH and $[Ca^{++}]$ in solutions in equilibrium with hydroxy apatite (27)

$$\log [Ca^{++}] = C - \text{pH}\,,$$

where C is an empirical constant. Levinskas, too in studying the solubility of L-apatite, found a linear relation (slope of unity) between pH and $[Ca^{++}]$ in the supernatant solutions (30) In both instances if the exchange substitution were *on* an equivalent-charge basis, one would expect $[Ca^{++}]$ to vary inversely with the *square* of the hydrogen ion concentration

This kind of indirect, empirical evidence is, of course inadequate. Unfortunately it is almost impossible to study pH as an independent variable. Every change in pH results in a corresponding change in pOH $[Ca^{++}]$ $[Ca^{++}]/[P_{total}]$ and $H_3PO_4/HPO_4^{=}$ Even a kinetic study of hydronium-calcium exchange with labeled ions (deuterium or tritium) is not practical because of the exchange of the label with the *whole* of the aqueous solution.

Hydronium ion substitution is equally unsatisfactory from the structural standpoint. The potassium ion which is only 40 per cent larger than calcium does not displace calcium in hydroxy apatite surfaces. How is it that 20 per cent of the total calcium positions in the lattice can be occupied by two hydronium ions (cf chap iii) each of which is at least 40 per cent larger than calcium? Perhaps it is not the hydronium but the hydrogen ion that actually participates in lattice exchange. The hydrogen ion, a bare proton occupies a negligible space and can diffuse through even a closely packed lattice. On statistical grounds, the crystal surface should have the composition $[Ca_2^{++}(PO_4^{\equiv})_3 OH^-]$/surface unit cell. Suppose under conditions of low pH and low Ca/P ratio the last layer deposited on the crystal had the following composition $[Ca^{++}H_2^+(PO_4^{\equiv})_3 OH^-]$ This can be rewritten as $[Ca^{++}H_3O^+(PO_4^{\equiv})_3]$ The basis for postulating the presence of hydronium in the lattice was the amount of water lost from low Ca/P apatites at elevated temperatures. Here it is seen that water loss can be explained as a loss of H^+ and OH^- by recombination

Of all the questions in the crystallography and surface chemistry of the apatite minerals hydronium ion substitution is the most perplexing and most difficult to resolve experimentally

Cations Inadequately Studied

Besides strontium radium and uranium, there are a number of other unphysiological cations which concentrate so selectively in the skeleton that they have been termed bone seekers." Unfortunately none of these has been studied sufficiently to permit an assignment of the underlying mechanisms but there do appear to be two distinct classes of cations based on their histological distribution in bone tissue. One group shows a distribution pattern very similar to that of Ca^{++} and includes beryllium and thorium besides strontium and radium. The other group concentrates in areas of active bone resorption and includes plutonium gallium yttrium and other rare earths, and some of the transuranic elements (31) As a class, this second group tends to form colloidal hydroxides. For a time it was thought that this group deposited in osteoid. This is not established however and plutonium at least, seems to have an affinity for bone mineral (19) Clarifying research on the mechanism of deposition of this second group of ions is urgently needed. Preliminary observations of the deposition of lead in a model system in our laboratory indicate that its entry into the crystal

does not involve a simple displacement of calcium as heretofore believed (44) This 'bone-seeker' needs reinvestigation.

ANIONS

Although calcium-displacing cations have received the most attention we know that both phosphate and hydroxyl ions can participate in crystal-solution interchange Recent studies have shown that certain physiological and non-physiological anions can compete in these interchanges.

Carbonate

One of the most important displacements is that of phosphate ions by carbonate ions, an exchange of both physiological and crystallographic interest The nature and state of the carbonate of bone mineral (up to 4 or 5 per cent CO_2 by weight) have been subjects of heated debate for nearly a hundred years and even today the problem is far from settled. There has been considerable clarification however and only two suggestions remain in serious contention (*a*) the exchange of carbonate for phosphate on the crystal surface (25 47) and (*b*) the substitution of carbonate for phosphate within the crystal lattice.

There still appears from time to time a third suggestion (50) that the carbonate is present as a separate phase of calcium carbonate. There is no critical evidence to support this idea, and it can be regarded as most unlikely Considered as support are two lines of evidence (*a*) the infrared absorption of carbonate in bone mineral is indistinguishable from that of calcite, $CaCO_3$ (49 59) and (*b*) an analysis of low angle X ray scatter (50) has been interpreted as indicating the presence of two sizes of particles one very small. While these two findings are consistent with the idea of a separate phase they do not exclude other explanations. Since the extracellular fluids are undersaturated with respect to the K_{sp} of $CaCO_3$ and sensitive X ray diffraction tests fail to demonstrate the presence of two phases, it seems quite unnecessary to propose that bone carbonate is present as calcite

Recent experiments, similar in design to those described previously for sodium have demonstrated that carbonate is removed by apatite crystals from solutions below the K_{sp} of $CaCO_3$ that phosphate is displaced from the crystals to the solution that the exchange substitution is readily reversible and that quantitatively this heteroionic exchange can account for the carbonate found in bone (47) More recently it has been shown that carbonate impregnation reduces the readily exchange-

able phosphate (both in the hydration shell and in the surface) of the crystals, as measured by $P^{32}O_4$ (57)

All these results fit the pattern characteristic of a surface limited ion capable of participating in both surface-charge neutralization and surface ion exchange. The extent of skeletal carbonate exchange in vivo closely parallels the extent of skeletal sodium exchange (cf chap. v), which is now accepted as being a surface limited reaction On the other hand in glycol-ashed bone the carbonate does not appear to be readily exchangeable or capable of easy removal by exposure to solutions of high phosphate content or low pH whereas sodium is. All this suggests that in bone mineral formed at only 37° C and from dilute solutions, carbonate is limited to the crystal surfaces, while in ashed bone and probably in geological specimens also the carbonate is situated within the crystal lattice. It is significant that most of the support for the 'intracrystalline carbonate hypothesis has evolved from the study of selected geological minerals (35) not from the study of fresh bone.

Citrate

While the presence of carbonate in bone mineral was recognized very early nearly a hundred years passed before Dickens discovered citrate to be an ever present constituent of the skeleton at about 1 per cent of the ash weight (16) Because of the importance of citrate in intermediary metabolism and its strong affinity for calcium ion its presence in bone raised a number of intriguing questions. Is there special significance to bone citrate? Is it adventitiously bound by the crystals by exchange with the body fluids, or does the citrate in bone originate from the metabolism of the bone cells?

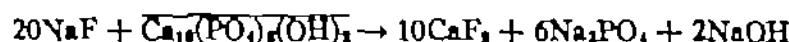
It has now been established independently by two different laboratories (3 58) that hydroxy apatite removes citrate from solutions very efficiently in vitro The mechanism of citrate-binding appears to be analogous to that for carbonate fixation—an exchange displacement of phosphate groups in the crystal solution interface. Because of its molecular size, it is improbable that citrate can participate in intracrystalline exchange rather of all ions it would be expected to be limited to participation in the neutralization of charge asymmetry in the hydration shell. Critical data on this point are lacking however The occurrence of this passive surface reaction appears to account quantitatively for the citrate content of bone (3) but this tells nothing of the origin of the citrate whether it is synthesized locally or is carried to the bone by the

circulation from an extra skeletal site of synthesis. Chapter vi deals with this problem in detail

Fluoride

The presence of carbonate and citrate greatly increases the solubility of hydroxy apatite (2) while fluoride markedly inhibits dissolution. It is this property of decreasing solubility that provides the basis for the use of fluorides in preventive dentistry It is also the probable basis for fluoride's chronic toxic effects at low levels of intake Thus physiological, mineral resorptive processes are inhibited by excessive dietary fluoride giving rise to mottled enamel, increased bone density skeletal malformation and exostoses.

There is some confusion in the literature concerning the mechanism of hydroxy apatite–fluoride interaction Much of this confusion results from the use in preventive dentistry of two quite different fluoride concentrations. For public water supplies, a fluoride level of 0.5–1 p p m is recommended Below this level, protection against dental caries is not maximal, and as the level is raised, dental fluorosis (mottled enamel) develops in increasing incidence. For topical application in the dentist's office, a concentration of 1–2 per cent is usually recommended. At the high concentration calcium fluoride CaF_2 is formed with a concomitant release of phosphate ions, a process analogous to double salt decomposition (21)



$$20NaF + \overline{Ca_{10}(PO_4)_6(OH)_2} \rightarrow 10CaF_2 + 6Na_3PO_4 + 2NaOH$$

This reaction in the mouth is probably limited to the surface layers of the dental enamel.

Parenterally such levels would, of course be lethal Fluoride deposition in the skeleton occurs at 1 p p.m. or less in the body fluids. The principal reaction at these levels appears to involve an exchange, which is not surface-limited, of fluoride for hydroxyl groups (45) It is not readily reversible in vitro (36) and very high skeletal levels of fluoride have been reported in vivo—too high a level to be accounted for as a surface reaction only (37) The fluoride exchange is extremely dependent on pH At alkaline pH only small amounts of fluoride are taken up by the solid and a steady state is quickly attained. At low pH much more fluoride is fixed by the crystals, and the system approaches equilibrium slowly Typical data (39) are given in Figure IV 15 All this is consistent with Grimley's mathematical analysis given earlier i.e. at high

[OH⁻] in solution there are few vacant hydroxyl positions in the crystal, and intracrystalline exchange is nearly abolished while at low [OH⁻] the fluoride ion easily diffuses into the crystal interior Nonetheless, clarifying research is needed.

Chloride

Like potassium the chloride ion diffuses into the hydration shell but does not concentrate there or undergo exchange at the crystal surface

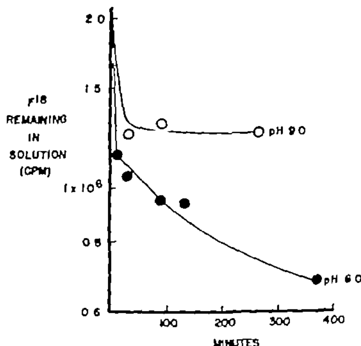


FIG IV 15 —The effect of [OH] on F^{18} uptake by apatite crystals. Conditions μ = 0 165 KCl = 0.160 M barbituric acid = 0.005 M pH adjusted with HCl or NaOH 25 mg L-apatite per 100 ml. Note near-absence of intracrystalline exchange (30-270 min) at pH 9 0 as compared with continued uptake at pH 6.0.

(56) Therefore, despite the high concentration of chloride in extracellular water its concentration in mature bone is quite low

Summary

It has been shown that all the possible chemical reactions which hydroxy apatite crystals might undergo involve a transfer of ions across the crystal-solution interface. The nature of this interface is not fully clarified at present, but it is certain that because the crystals are so

tiny and calcium is non polarizable strong electric fields are projected away from the crystal surfaces. These fields give rise to a bound ion layer which in turn acquires an insulating layer of water—the hydration shell. Exchange between ions in solution and those in the hydration shell is extremely rapid. Exchange between ions in the hydration shell and the crystal surface is fairly rapid but is dependent on the rate at which the ion is thermally ejected or diffuses out from its lattice posi

TABLE IV-6

SUMMARY OF PRESENT EVIDENCE CONCERNING PARTICIPATION OF IONS IN HYDROXY APATITE-SOLUTION INTERACTION

Ion	Penetration of Ion into: Hydration Shell, Water Only	Hydration Shell, Bound Ion Layer	Crystal Surface	Crystal Interior	Ion Displaced
K^+	+	−	−	−	−
Na^+	+	−	+	−	
Mg^{++}	+	+?	−?	−	
UO_2^{++}	+	+	−?	−	
Sr^{++}	+	+	+	+	Ca^{++}
Ra^{++}	+	+	+	+	
Ca^{++}*	+	+	+	+	
Cl^-	+	−	−	−	−
Citrate$^{\equiv}$	+	+?	−?	−	
$CO_3^=$	+	+	+	−	$PO_4^{\equiv}$
$P^{32}O_4^{\equiv}$	+	+	+	+	
F^-	+	−?	+	+	OH^-

* Not studied directly: deduced from physiological data (7).

tion. Exchange within the crystal is quite slow and is dependent on the thermal migration of defects and vacant lattice positions. This thermal intracrystalline exchange can be described in only semiqualitative theoretical terms at present.

Presumably any ion present in solution can and will penetrate the hydration shell. Only specific ions tend to concentrate in the hydration shell. A greater and different specificity is required of ions penetrating the surface, and of these, only a few are sufficiently similar to normal

lattice ions to permit entry into the crystal interior The status of our knowledge concerning the differential distribution of various physiological and bone-seeking foreign ions is summarized in Table IV-6. There is great need for additional research on the nature of the hydration shell, on the kinetics of Ca^{45} exchange, and on the mechanism of apatite crystal interactions with a number of cations—H_3O^+ Mg^{++} Pb^{++}, and the rare earths in particular

Bone mineral in vitro shows exchange properties similar to the synthetic hydroxy apatite model system varying from less active in established cortical areas to much more active in areas of new crystal deposition.

CHAPTER V

Skeletal Dynamics

We have now reached the stage in our development of concepts of bone metabolism where we must leave the test tube and turn to the animal as the container for our experiments. We have pointed out from time to time that, for lack of theoretical background and critical data we were skating on very thin ice even in the interpretation of results obtained with simplified model systems in vitro From here on the skating becomes even more hazardous. Indeed in certain areas we must walk on water or drown in confusion

Briefly the problem is to project the rules of behavior of the aqueous-apatite crystal system derived from equilibrium studies into the dynamic whirlpool of animal metabolism with its built in checks and controls, where change is the order of the day, where equilibrium is a goal ever sought but rarely achieved. Our problem is very much like that of the honest politician who would attempt to apply the Golden Rule to practical politics.

The Water of Living Bone

One thing is certain to extrapolate from laboratory studies of isoionic and heteroionic exchange in hydrated crystals in vitro one requires an intimate knowledge of the crystal-solution interface in living bone itself. It is here that we meet our first disappointment only a half dozen papers in the entire literature are concerned with the state of water in bone.

Two publications by Deakins (29 30) now nearly fifteen years old, are classics, however and these will be discussed in some detail The tissue selected for study was unerupted dental enamel (pig) a wise choice for it is just about the only calcified structure that is histologically homogeneous. Density wet weight dry weight, and ashed weight were determined on each sample. Reasoning that the expression of composition can be misleading the author chose to report his data as weight per unit volume (mg/mm^3) A summary of these data is given in Figure V 1

Deakins work was performed with great care and attention to detail Unfortunately, the *significance* of his findings went unnoticed throughout the succeeding years. Only recently has the importance of volume considerations as contrasted with weight considerations been

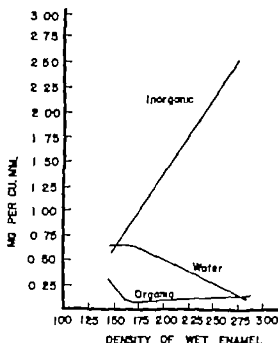


FIG. V 1 —"Summary of chemical changes in developing pig enamel at various densities. The slope of the ash curve is 1 59 the second part of the water curve, −0.5 1.59/3 18 = +0.5 or slope of a curve which would be the rate of increase in volume of ash per cubic millimeter of enamel. (Weight divided by density is equal to volume.) These two slopes being equal and opposite in sign means that the volume of ash deposited just fills the space from which the water was displaced. 3.18 is the accepted density of apatite, the mineral substance in enamel" (79)

rediscovered. This rediscovery has been prompted by two developments (*a*) chemical evidence that the water of calcified tissues is largely in a bond form and (*b*) morphological (and turnover") studies showing that the organic phase principally collagen is a fixed quantity suggesting a reciprocal relation between the crystals and the water present

Deakins conclusion that the *volume* of ash deposited just fills the

space from which the water was displaced" is of fundamental importance. It suggests that the driving force toward mineralization inherent in the supersaturated body fluids is so great that crystallization proceeds until there is no space left for further expansion and the water is nearly all displaced. It might be asked why *all* of the water is not displaced. This can be answered only in terms of rates. As crystals form and grow in a fixed volume by displacement of water, the water spaces between crystals or between crystals and adjacent protein fibers become smaller and smaller. For the crystals to grow, ions must diffuse in from the circulation. As these water spaces approach atomic dimensions, ions can no longer diffuse at appreciable rates. This is easily seen in Figures V 2 and V 3, taken from Sollner (78), who discussed the restriction of diffusion by charge and pore size. The diagrams represent the case for univalent ions and charges. It is of special interest to us here concerned with Ca^{++} and $PO_4^{\equiv}$ that Sollner states: "Polyvalent ions . . . are much more restricted in their permeation . . . than univalent ions because polyvalent ions are large and have a high charge that prevents them by electric repulsion from entering narrow pores which are accessible to univalent ions."

From Deakins' data and corollary information, we can calculate the thickness of the water space in maximally mineralized enamel, provided that certain reasonable assumptions are permitted. These calculations, in which all doubtful assumptions are designed to maximize the water space, are given in Table V 1, showing that in fully mineralized enamel the water spaces are, indeed, approaching atomic dimensions. Crystals cannot continue growing if ions cannot diffuse in from the outside environment.

This theoretical development is based on data obtained from the study of enamel. What of bone? Robinson has reported (75) that the volume analysis of Deakins can be applied also to bone, but there are some uncertainties introduced because of the histological heterogeneity even of selected samples of cortical bone. Robinson has attempted to account for the water content of the osteocytes, canaliculi, and Haversian canals and, to the consternation of many, has found that the total water present in cortical bone is only sufficient to fill these spaces, leaving no water at all for the crystals or the organic phase in fully mineralized bone! But his calculations must be subject to an uncertainty of 3–5 per cent. For theoretical reasons given above, crystallization cannot exclude *all* water. In support of Robinson's contention is the finding

that little or no water can be removed from finely powdered cortical-bone specimens young or old by enormous centrifugal forces—forces sufficient to strip from the crystals all mechanically held water (64)

Fortunately Robinson's findings can be confirmed within the error likely in his measurements by calculations which are based on the quantitative microradiographic results of Amprino (2) who found that various Haversian systems exhibit varying densities. Some density variation is seen even within a single Haversian system. Older or primary interstitial lamellae are the most mineralized, having a maximal

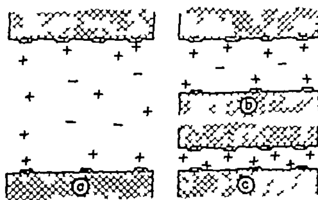


FIG. V 2.—"The distribution of ions in three pores of different diameter at the same concentration of the outside electrolyte solution" (78)



FIG. V 3.—"The blockage of the passage of ions by charges of the same sign that are fixed at the walls of a pore. (*a*) Blockage by one fixed charge in a narrow pore. (*b*) The influence of the relative location of several fixed charges at the walls of a wider pore. The continuous circles around the plus or minus signs represent here the size of the ions, including their effective shell of hydration, the dashed circles indicate the effective range of the electrical force of repulsion acting at the given ionic strength between the fixed charges situated on the pore walls and ions of identical signs in the solution. The tip of the arrow indicates how far the ions identical in sign with the charged wall group may move from the left side" (78)

nsity of about 2.40.[1] From this it may be calculated as in Table V 2 that these areas of maximally mineralized bone contain only about 3 per cent water, an amount only twice that found by direct measurement in enamel. Since greater amounts of organic material are present in bone and since the crystals of bone are smaller than those of enamel, it is safe to conclude that, as with enamel, maximal mineralization of

1 Actually Amprino's results are expressed only as relative densities. However, according to R. A. Robinson, Amprino's relative densities may be regarded as very close approximations of the absolute densities.

TABLE V 1

CALCULATION OF WATER SPACE OR WATER FILM THICKNESS IN MAXIMALLY MINERALIZED ENAMEL

Assuming that

Maximal thickness of water film will be given if *all* water present is assumed to be on crystal surfaces, none in interstitial spaces, and the organic matter is not hydrated.

Maximal thickness of water film will be given if largest value for effective area of water molecule is assumed: 10.5 A²

Maximal thickness of film will be given if one of the large surfaces of crystal is assumed contiguous with an organic fiber leaving only half the crystal surface available for binding water

Since specific surface measurements are not available for enamel (free of organic material) a value must be calculated from the observed crystal thickness of 100 A (87) (see chap. iv, Table IV 1, crystal No. 8) or 100 M²/gm.

Given (29) Maximum density of enamel = 2.76, Mineral = 2.62, organic matter = 0.05, water = 0.12, all as gm/ml of enamel.

I. Surface area/ml enamel = Grams/ml × surface area/gm

II. Surface area exposed/ml = Surface area/gm × ½

III. No. molecules of water to make monolayer/ml = $\frac{\text{Surface area exposed/ml}}{\text{Surface area}/H_2O\text{ molecule}}$

IV. No. of H_2O molecules present/ml = $\frac{\text{Wt. of water/ml}}{\text{Molecular wt}}$ × Avogadro's No.

V. No. of molecular layers = $\frac{\text{No. }H_2O\text{ molecules/ml}}{\text{No. }H_2O\text{ molecules in monolayer}}$

I. 2.62×100 M²/gm = 262 M² = 2.62×10^{22} A²/ml

II. 2.62×10^{22} A²/2 = 1.31×10^{22} A²/ml

III. $\frac{1.31 \times 10^{22}\text{ A}^2}{10.5\text{ A}^2}$ = 1.25×10^{21} molecules/ml

IV. $\frac{0.12}{18} \times 6 \times 10^{23}$ = 4×10^{21} molecules/ml

V. $\frac{4 \times 10^{21}}{1.25 \times 10^{21}}$ = 3.2 molecular layers

plete mineralization Thus, as the osteoid for a new lamella is laid down, it rapidly mineralizes to the point at which diffusion rates begin to fall. Thereafter it very slowly approaches full mineralization In terms of the life span of a lamella then only a small time interval is devoted to the accumulation of the *bulk* of the mineral. Actually, this has been the experience of the histologist employing the microradiographic technique (4 8 70) In examining a section of bone he finds few if any Haversian systems or trabeculae primary or secondary with a low degree of mineralization even in very young bone. On the

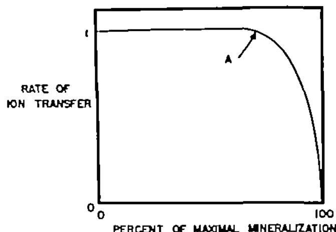


FIG. V-4.—A diagrammatic representation of the relation to be expected between rate of ion transfer (exchange, mineral acquisition, or dissolution) and the degree of mineralization of any given structural element of bones or teeth. A rate of unity is the maximal rate of unimpeded diffusion. At point *A* the water spaces are approaching atomic dimensions, thus restricting diffusion rates.

other hand, he finds a great many bone elements which are almost (80–90 per cent) but not quite, fully mineralized.

The rates of diffusion of ion transfer and therefore of mineralization should continue maximally up to a certain critical water content. Thereafter they should fall precipitously with each small increment in the degree of mineralization as presented diagrammatically in Figure V-4 That these expectations are realized in the intact animal is indicated by the studies of Cartier (23) which are illustrated in Figure V 5 At the histological level this phenomenon has been demonstrated by the use of quantitative radioautography (10)

It is not known at present whether the increase in mineralization is due only to crystal growth or whether new crystals also form Probably

both occur but the gross effect is the same in either case. There is evidence that older crystals are larger, as seen in the electron microscope (87) It has been shown that the ratio in bone of certain surface-limited ions to calcium falls with increasing age of the rat (71) an observation to be expected if crystal size increased causing the ratio of surface ions to interior ions to fall. Another bit of evidence can be drawn indirectly from radon excretion The proportion of exhaled radon derived from skeletally deposited radium falls with passing time (68) Since radium resides within the crystal (chap iv) and since radon escapes by virtue of its recoil energy derived from the disintegration of radium (a con-

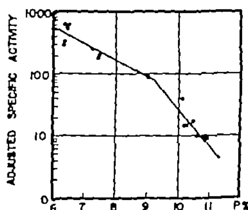


FIG. V 5 —Data illustrating the sharp decline in diffusion rates (as indicated by skeletal deposition of injected $P^{32}O_4$) with increasing degree of mineralization (as indicated by the weight percentage of phosphorus in bone) Taken from Cartier (23)

stant) this means that the effective mass through which the radon must blast its way is increasing This can be interpreted to indicate crystal growth (68)

Another factor to be considered in the mineralization process is the organic content. Water is displaced collagen is not (29 30 75 81) The maximal degree of mineralization is obviously governed by the *amount* of organic material present the less organic matter in a bone or tooth structure, the higher the degree of mineralization possible, as diagramed in Figure V-6. For example, the high-density lines—the so-called cementing lines —circumscribing Haversian systems are not rich in mineral by virtue of the nature of the organic matter present but rather by virtue of the *lack* of organic material in these areas. This is a

histologically demonstrated fact (25) This same relation has been demonstrated in cartilage (74)

Because of the heterogeneity of bone at the histological level it should be fairly obvious that values for the water content obtained by drying whole bones have little meaning with respect to the hydration of the mineral crystals in vivo This is especially true if care is not exercised in completely excluding marrow and other soft tissue from the samples to be analyzed a difficult task with cancellous bone For the sake of completeness only the range of such analyses will be listed cortical bone from 10 to 22 per cent cancellous bone from 30 to 52 per cent (34 73) The data calculated and observed presented in this

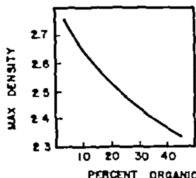


FIG V-6.—A calculation of the relation between maximal density and organic content derived from Table V 2 The water mineral ratio is kept constant at 0 04 ml H_2O/0.300 ml inorganic.

chapter suggest that of the cortical bone water about 5–10 per cent is located in blood vessels and osteocytes and their accompanying lacunae and canaliculae much of the remainder must be regarded as bound hydration shell water

The data of Edelman *et al* (34) on the exchange of cortical-bone water as measured by deuterium indicate that bone water is 100 per cent exchangeable. Because water dissociates (in this case $D_2O \rightleftharpoons D^+ + OD^-$) this study describes the permeability of bone to the ion D^+ The laboratory pH meter employs a glass electrode proof that the proton H^+ can permeate even glass. While the deuteron may not be so agile as a proton it should easily permeate all areas of bone Actually the exchange was not complete even in 4 hours (only 90 per cent) while most soft tissues equilibrate in a few minutes. This is an indication of restricted diffusion of even the deuteron.

PATTERNS OF SKELETAL REACTIVITY

We have defined ion exchange in our model systems in vitro as an equilibrium condition in which there is no net gain or loss between solid and solution phases. In the living skeleton where there is continuing acquisition of mineral it is probably incorrect to use the term "ion exchange" to describe ion transfers back and forth across the crystal-solution interface. However for every ion *added* to the crystal, hundreds thousands, perhaps millions, of ions have *exchanged* therefore the term ion exchange is descriptive even if slightly inaccurate. In any event, the four physicochemical processes elucidated with model systems in vitro—(*a*) diffusion into the hydration shell (*b*) exchange in the crystal's bound ion layer (*c*) exchange at the crystal surface, and (*d*) intracrystalline exchange—are all operative in the living skeleton but are markedly modified by in vivo conditions.

Because the serum is supersaturated with respect to calcium and phosphate, the tendency is for all bone structures gradually to attain maximal mineralization The more fully mineralized the structure, the more restricted are the circulation diffusion, and exchange of ions. From this it follows that the *age* of the bone is the primary determinant of its chemical reactivity This applies to any bone structure, whether it be a trabecula a Haversian system, an interstitial or subperiosteal lamella. Young bone is more vascular and has a higher water content, permitting fast diffusion rates and a high rate of acquisition of additional mineral besides the crystals, being small and less perfect, permit rapid surface exchange recrystallization,[2] and intracrystalline exchange (3 4 6 10 15 31 37, 52 57 86) However the continual acquisition of mineral at any level of development imparts an irreversibility to all exchange reactions in vivo. Those ions which can migrate into the lattice are soon buried under layers of subsequently added mineral with little possibility that they will migrate back to the surface and escape before the crystal becomes diffusion locked. Even a surface limited ion like sodium can be trapped. As the water content falls to its minimal value, the sodium ion can no longer freely diffuse back to the circulation This irreversibility of exchange in vivo was recognized early (63) but was not chemically demonstrated until much later (58–60) The rapidity with which irreversibility develops is only now fully appreciated

2. The term "recrystallization" is used here in the conventional, classic sense the dissolution and redeposition of crystals

These two factors governing skeletal reactivity in vivo—(*a*) the inverse relation between the age of the bone element and the rates of ion transfers and crystal formation and (*b*) the irreversibility of exchange reactions because of intracrystalline exchange and crystal growth—permit the use of radioactive ions and dyes which chemisorb on apatite crystals, as excellent histological markers for the study of the growth processes in bone (53) The bulk of the injected dose quickly concentrates in the areas of current and recent sites of bone formation. After a short time, the marker is irreversibly fixed and undergoes very little translocation. In time of course, an old growth site may be actively resorbed but in such a case the release of the marker is gradual and it becomes so diluted in the general circulation that no appreciable error (53) of translocation occurs in the histological picture

Thus the crystal-seeking radioactive ions (irrespective of size or charge) concentrate in the growth areas, giving characteristic autoradiographic patterns, depending on the species and age of the animal and the bone or area of bone studied This is illustrated for a long bone in Figure V 7 In the young bone Figure V 7 1 the highest concentrations of isotope are seen in the growing ends at the epiphyseal plate, on the endosteal surfaces in the funnel and on the outer periosteal surfaces of the shaft. These correspond exactly to the sites of most rapid remodeling and appositional growth Not clear in a gross autoradiogram but easily seen at higher power (Fig V-8) is the diffuse radioactivity in pre-existing bone. Later in the animal s life, as growth rate subsides and the pre-existing compacta becomes more fully mineralized a very sharp autograph is obtained. The radioactivity is found in the same general areas the epiphyseal plate the endosteal funnel and the subperiosteal layer However the lines of deposition are thinner and more sharply defined. Much less diffuse activity is found in pre-existing compacta. In the non-growing adult bone (Fig V 7 *B*) there is still less diffuse activity the former growth areas are relatively inactive and only small local sites of intense radioactivity are seen more in cancellous bone surfaces but also patches in the compacta. At the histological level these patches, varying in number with the species, correspond to the recently formed or forming Haversian systems (10) of low density as illustrated in Figure V 9

The decline in growth and growth remodeling with advancing age results in an ever increasing proportion of the skeleton that is old fully mineralized and unreactive This is illustrated diagrammatically in

Figure V 10 The presence in the animal of a large portion of mineral phase which seems to be practically unreactive has given rise to a variety of terms to describe it "stable bone," "unavailable skeleton," "non-exchangeable bone" etc. None of these terms carries histological implications, although it is realized that probably the bulk of the unreactive skeletal mass is in the compacta (10) We shall use the terms "unavailable" and "available skeleton," but it should be stressed that

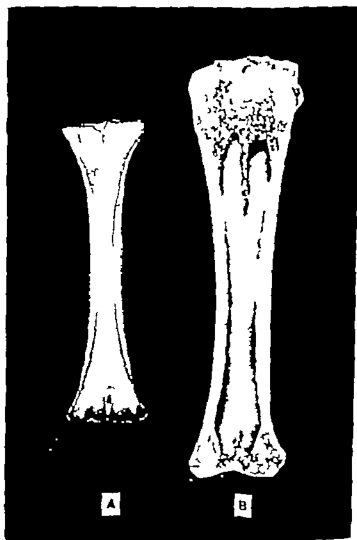


Fig V 7 —Representative autoradiograms of metatarsals of cattle 7 days after administration of Ca^{45} *A* 1 month-old calf *B* 1 year-old calf Courtesy C. L. Comar and associates.

this arbitrary division lumps into the "available" category a variety of structures with an infinite variety of exchange rates and degrees of availability

That the underlying mechanisms of fixation are passive physicochemical processes is emphasized by the fact that they occur also in non vital sections of calcified tissues in vitro even after microincineration (5) The newer less calcified structures show greater uptake as they do in vivo (5), and as in the animal the histological pattern of distribution is the same whether the isotope employed is Ca^{45} (5 15) uranium (6) $P^{32}O_4$ (15 36) or Sr^{89} (72) The physiological state of the tissue determines only the *degree* to which these physicochemical phe-

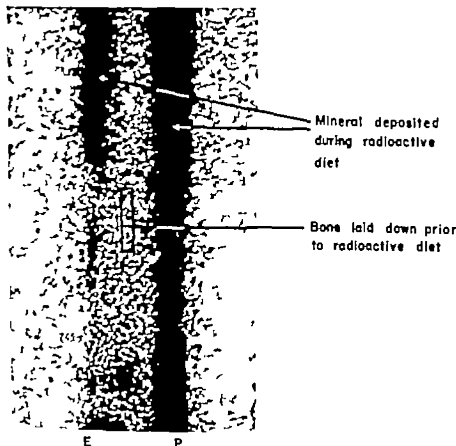


FIG. V-8.—A radioautograph of the shaft of the femur of a young adult rat placed on a radioactive diet (Ca^{45}) *E* and *P* denote endosteal and periosteal surfaces. Note the heavy deposition in the compacta which had been laid down before inception of the radioactive diet Taken from Tomlin *et al* (85)

A

B

FIG. V 9.—Comparative photographs of *A* radioisotope deposition, and of *B* opacity to soft X rays. Note the correlation between isotope concentration and areas of low density. Taken from Amprino (7)

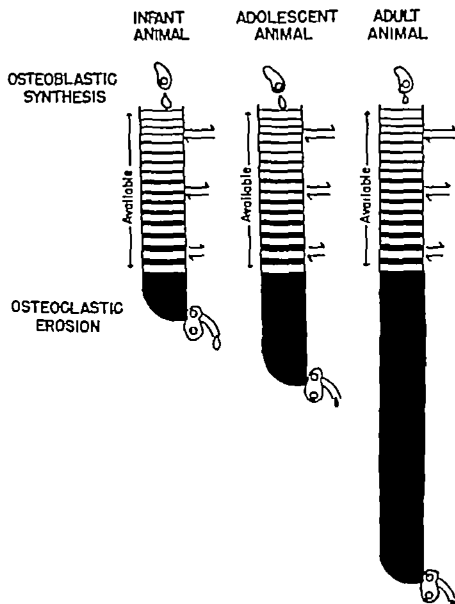


FIG V 10 —A diagrammatic representation of the changing skeletal reactivity with age. Arrows represent ion-transfer rates In the infant most of the bone is new hydrated, and therefore available new growth and growth remodeling occur at a rapid pace In the adolescent, growth and growth remodeling have slowed down, much of the skeleton is fully mineralized and inert. In the adult, the bulk of the skeleton is fully mineralized and unreactive. Though the proportion of the total skeleton which is available decreases with age the total available pool probably remains essentially the same (39)

nomena can occur and in the animal the *degree* of irreversibility of these processes.

Haversian remodeling in compacta seems to correlate with the evolution of the higher vertebrates (38) What evolutionary advantage is served by this intricate process? Does it increase structural strength (43)? Hardly, but it may insure continuing strength. Strain points or structural cracks are obviated because the tangle of interweaving osteones renders impossible the development of the fault lines that we occasionally see in man-made structures. But, apart from structural considerations, can remodeling serve a metabolic function? It may (1 51, 76) We have described the process of continual acquisition of mineral by all bone elements. What would happen to the skeleton of an adult animal a few years after growth had ceased? Would not practically the entire skeletal system become fully mineralized and thus dif fusion-locked and metabolically inert? Later we shall consider the participation of the skeleton in electrolyte regulation. This important metabolic function would be lost to the adult animal if Haversian remodeling did not insure a continuing fresh supply of new and reactive bone (51) In this connection there is an interesting indication that Haversian remodeling increases as the growth rate of the animal decreases (76)

Attempts To Untangle the Physiological Snarl

As long as we deal as in the preceding section, with the general skeletal patterns of ion exchange and ion fixation in qualitative terms, a coherent story can be given. However it is our great hope that radioactive isotopes will give us still more detailed information on the quantitative relationships of the various processes involved in the mineral metabolism of the skeleton. There is a great need for means by which the metabolic defects of the various diseases of bone can be isolated and defined. Only in this way can we aspire to develop rational measures for diagnosis and therapy The operational plan, of course, is to administer a radioisotope to the normal man and animal and then to develop a means of analyzing the resulting data in such a way as to quantitate the rates of the various metabolic processes, and, finally to apply this information to various disease states. At the present time, no one has successfully analyzed data obtained even from the normal animal.

The reason, of course is the complexity—the physiological snarl—of interrelated events occurring in the skeleton The various ways a labeled ion may enter the mineral phase of the skeleton subject to physio-

logical influences may be listed (we are presuming it can diffuse to the site) as follows

I Physicochemical mechanisms

a) The formation *de novo* of crystals on newly synthesized organic matrix (NEW-CRYSTAL FORMATION)

b) The recrystallization (in the classic sense of dissolution and redeposition) of recently deposited crystals (RECRYSTALLIZATION)

c) The exchange for an unlabeled ion in a pre existing crystal surface (SURFACE EXCHANGE)

d) The diffusion of an exchanged surface ion into the crystal interior (INTRACRYSTALLINE EXCHANGE)

e) By virtue of the serum s supersaturation the continuing growth of crystals in established calcified structures (CRYSTAL GROWTH)

II Physiologically modifying influences

a) The synthesis of new endochondral cartilage and new osteoid in growth and remodeling (MATRIX FORMATION)

b) The resorption of previously calcified bone and cartilage in the growth process (growth remodeling) and in Haversian remodeling (RESORPTION)

c) The age of the structural element which determines the rates of diffusion and recrystallization the extent of intracrystalline exchange and of crystal growth (MATURITY-MATURATION)

d) General regulatory factors. In the normal animal excretion rates dilution of circulating isotope by absorption of dietary unlabeled material normal parathyroid regulatory action etc., will influence rates Any abnormalities in vitamin D level dietary intake parathyroid activity etc. are therefore important controlling variables

Limiting ourselves to the normal animal and to short time intervals after administration of the isotope to minimize the effects of the general variables, we easily see that all the physicochemical mechanisms are interrelated and as discussed above are dependent upon the age of the bone structure under examination Taking the skeleton as a whole the degree of reactivity in the normal animal depends solely on II*a* the rate of matrix formation The interdependency of the various mechanisms and modifying influences makes this the prime variable. Thus I*a* I*b* and II*b* depend directly upon II*a* Also I*c* I*d* and I*e* depend directly on II*c* which in turn depends on II*a* Even with this simplification the heterogeneity of the skeleton is such that structural units at all stages of development are always present Thus the rate of skeletal incorporation of an injected radioisotope is an over-all average of an

infinite array of rate processes varying from instantaneous to extremely slow most of which are more or less irreversible (10)

The earliest attempts to analyze data obtained by means of isotopes in terms of rate of renewal of the skeleton have been reviewed by Hevesy (48) The more recent attempts to develop useful systems of data analysis will be discussed here.

ISOTOPE DILUTION CONCEPT

One of the simplest applications of a radioisotope to the study of bone metabolism is the utilization of the concept of isotope dilution. If one assumes that two compartments are in equilibrium then $SA_1 = SA_2$ where SA is specific activity in counts per minute per milligram. By determining the ratio SA_{bone}/SA_{serum} one might hope to estimate directly the fraction of bone that is in rapid equilibrium with serum. We have available or can obtain labeled phosphate, calcium sodium, hydrogen hydroxyl carbonate, and citrate Unfortunately the last four cannot be used in vivo for our present purpose because of problems of extreme dilution, rapid exhalation or rapid metabolism. Attempts have been made to employ radiophosphate and radiocalcium but it has been the general experience that no equilibrium point is ever reached in vivo Because of the irreversibility of recrystallization, intracrystalline exchange crystal growth and new-crystal formation, the ratio SA_{bone}/SA_{serum} continually rises and ultimately exceeds unity Actually the SA of bone rises to a plateau, while the SA of serum continues to fall with passing time There are practical applications of this ratio under special experimental circumstances, and these will be discussed subsequently However for the straightforward application of the concept of isotope dilution following a single injection of isotope, radiocalcium and radiophosphate are not useful.

The only remaining labeled physiological bone-seeking ion is radiosodium. Fortunately the necessary assumption of a two-compartment equilibrium appears to be reasonably valid for radiosodium. For example, the work of Armstrong *et al* (9) may be cited. In 2 hours the SA of the blood is essentially stabilized, as is also the SA of bone As discussed previously (chap iv) sodium is a surface limited ion and cannot participate in recrystallization or intracrystalline exchange. Nor can it be buried by the acquisition of additional mineral during maturation. It can be trapped of course, as the unit of structure approaches full maturity and this probably accounts for the "unavailable" sodium of bone This process is quite slow however and, over a 2–24-hour period,

the exchange of sodium in the available skeleton can be considered completely reversible for all practical purposes. Thus the simple ratio SA_{bone}/SA_{blood} at 2-24 hours after administration of radiosodium pro vides a simple and fairly precise measure of the available mineral sodium in the bone taken for analysis. Whether this is a valid measure of the available skeleton itself depends on how representative the sample is. A number of such studies have been made and the observed results, in terms of exchangeable bone sodium vary from 80 per cent in very young to about 30 per cent in adult animals (cf Table V 5 and Fig V 12) As expected the percentage of available skeleton is directly related to growth rate It is perhaps surprising that only 20 per cent of the skeleton of a weanling rat has fully matured It is definitely surprising that the adult rat possesses a skeleton 30 per cent of which is "young" New bone formation resorption, and remodeling must occur at a rate which has frequently heretofore been grossly under estimated.

This use of radiosodium has provided much useful information on gross skeletal reactivity in animals. From the clinical standpoint it is of limited value however Biopsy or autopsy material is required a few hours after isotope administration. One might ask why this awkward situation cannot be circumvented by merely calculating the dilution of the radiosodium in the blood after correcting for the extracellular sodium. Here the difficulty is a matter of distribution Roughly one third of the body sodium is in the skeleton and in the adult about one third or less of this is available or $0.3 \times 0.3 = 0.09$ Assuming that one can estimate extracellular sodium with about a 10 per cent error (0.66 ± 0.066) the error in evaluating the contribution of exchangeable bone sodium would be enormous (0.09 ± 0.066) It s hopeless!

What is needed here is a relatively non toxic ion which is surface limited like sodium but unlike sodium is rapidly cleared by bone from blood and the extracellular fluids. Ideally it should have a short lived, easily measured radioactive isotope.

KINETIC ANALYSIS

Recognizing the difficulty in attaining an equilibrium in vivo a number of workers have attempted to perform a kinetic analysis of the time course of the disappearance from the circulation of an injected radioisotope Almost invariably the curves of radioactivity in blood or serum versus time have been expressed as a composite of exponential decay curves. This standard method of curve analysis involves an

extrapolation of the last "linear" portion of the over all curve (semilog plot) subtraction of this from the early part of the curve, and repetition of the process with each new calculated curve as often as necessary to reach zero time. The method is fraught with uncertainties. It is frequently said with some justification that any curve can be expressed as a series of exponentials. It is necessary therefore to support such an analysis with corollary data. One obtains by this method

$$C(t) = Ae^{at} + Be^{bt} + Ce^{ct} \quad Ze^{zt},$$

where C is the concentration of the radioisotope in serum or plasma at time t the exponents represent rates and the coefficients are compartment sizes. It is a difficult problem to be certain that each exponential represents a physiological process and not just an arbitrary subdivision of a lot of overlapping rate processes.

One real advantage of the method is the large number of radioactive ions that can be studied Any radioactive ion that selectively deposits in the skeleton is suitable—the more selectively, the better

In 1954 Thomas, Litovitz, Rubin and Geschickter (83) determined the Ca^{45} levels in plasma for 6 hours following an intravenous injection and expressed their results as a series of four exponentials

$$\text{Adult rabbit } C(t) = 0\,483e^{-1\,[illegible]t} + 0\,26e^{-0\,177t} + 0\,113e^{-0\,[illegible]t} + 0.144e^{-0.[illegible]t} \quad \text{(I)}$$

$$\text{Young rabbit } C(t) = 0\,448e^{-1\,[illegible]t} + 0.31e^{-0\,[illegible]t} + 0\,166e^{-0\,[illegible]t} + 0\,074e^{-0\,[illegible]t} \quad \text{(II)}$$

where $C(t)$ is the percentage of the injected dose in the plasma at time t These authors also determined the uptake of isotope by the femurs and obtained the following relation

$$\text{Adult } B(t) = 0\,023 \quad (1 - e^{-0\,[illegible]t}) \quad \text{(III)}$$

$$\text{Young } B(t) = 0\,05 \quad (1 - e^{-0\,[illegible]t}) \quad \text{(IV)}$$

From these results and other considerations, it was concluded that the first two exponential terms in equations (I) and (II) are governed by rates of "mixing time" transcapillary exchange into extravascular fluid soft tissue pickup etc. The third term was ascribed to bone uptake and the fourth to excretion From our previous discussions it appears questionable that a single exponential term can accurately describe even the averaged rates of all the skeletal processes. Actually as determined on femur (83) and on long bones (63), the bone uptake

curve is not truly exponential in form and there is reason to believe that such samples are not representative of the skeleton as a whole (71) It is surprising but hope-inspiring then that the averaged rate as approximated by the exponent was the same in both young and old animals whether the exponent was derived from the blood curve or the "bone curve"

Also in 1952 Armstrong and collaborators published a similar exponential analysis of the blood disappearance curves of both Ca^{45} and Na^{22} using dogs (9) While these authors did not definitely ascribe the third exponential term to skeletal reactions, such can be inferred and their results were gratifyingly similar to those of Thomas *et al*

$$\text{Calcium, } C_p = 340e^{-[illegible]} + 212e^{-[illegible]} + 105e^{-[illegible]} + 102e^{-[illegible]}$$

$$\text{Sodium, } C_p - 163 = 319e^{-[illegible]} + 188e^{-[illegible]} + 80e^{-[illegible]}$$

where C_p is given in counts per minute per milliliter Only three terms were required to describe the Na^{22} data.

Such blood disappearance curves deserve further study in experimental animals where comparisons can be made with bone uptake because, if the technique should prove useful, it could easily be developed into a routine clinical procedure. A major advantage is that no biopsies are required. However at the moment the likelihood is poor that this approach will develop clinical usefulness. In a later study Thomas, Litovitz, and Geshickter (82) demonstrated that the coefficient and exponential term (ascribed to bone) was markedly influenced by the level of calcium in the gut Since large variations in skeletal metabolism (young versus old) (83) showed smaller changes in this term, it is obvious that the third term is not uniquely determined by the metabolic state of the skeleton. Furthermore, Arnold, Jee, and Johnson (10) claim that Ca^{45} deposition in established bone is nearly complete in 1 hour If such is the case the first and second exponential terms must also contain significant contributions from skeletal uptake It would appear then that deposition rates in the skeleton varying from extremely fast to extremely slow overlap hopelessly other non-skeletal rates, such as mixing time absorption, and excretion.

A few analyses of Ca^{45} disappearance from the blood in man have been made, but as yet, the short term disappearance from the blood has not been studied adequately Despite the animal studies just described few if any determinations were made in the early time intervals (16 21 50) These curve analyses are concerned with events which

take place *after* the bulk of skeletal deposition of isotope has occurrec events such as bone resorption, dilution by dietary calcium and urina and fecal excretion etc. One report (30) calculated calcium "pools" using the concept of isotope dilution. The underlying (not so stat assumption in such a calculation is that the system is completely versible, i.e. $SA_{serum} = SA_{pool}$, where SA represents specific activi Since this assumption is invalid after 2 hours when Ca^{45} is employ (9) all such calculated 'pools' are of doubtful significance.

THE BAUER, CARLSSON LINDQUIST ANALYSIS

The kinetic approach (exponential analysis) has thus far not p vided much useful information on bone even in experimental anima As a variant of this approach, Bauer Carlsson and Lindquist (have attempted the use of a simple mathematical device for divid the radioactivity found in bone after a single injection of isotope h three fractions. Their assumptions and development are quoted he

$$Ca^{45}_{Obs} = Ca^{45}_{E} + Ca^{45}_{A} - Ca^{45}_{R} \qquad ($$

Ca^{45}_{Obs} = total amount of Ca^{45} present in a calcified tissue.

Ca^{45}_{E} = amount of Ca^{45} present in the exchangeable fraction of the bone salt

Ca^{45}_{A} = amount of Ca^{45} incorporated into the non-exchangeable fraction of the bone salt through accretion.

Ca^{45}_{R} = amount of Ca^{45} removed through resorption

Under the assumption that the specific activity

$$\frac{Ca^{45}}{Ca^{45} + Ca^{40}}$$

of the exchangeable Ca fraction rapidly attains the same value as the spec activity of the plasma Ca and that these two specific activities can then regarded as equal we get

$$Ca^{45}_{E} = E \times S \qquad (:$$

E = amount of Ca ($Ca^{40} + Ca^{45}$) in the exchangeable fraction of the bone salt.

S = specific activity of the serum (plasma) Ca.

Since it must be assumed that Ca^{45} and Ca^{40} are deposited in the bone salt in the same proportions as they simultaneously occur in the blood we get

$$Ca^{45}_{A} = A \times T \times S_{M} \quad (3)$$

A = rate of Ca accretion

T = interval of time between administration of Ca^{45} and observation

S_M = *average* specific activity of serum Ca during this time interval

Combination of equations (1) (2) and (3) gives

$$Ca^{45}_{Obs} = E \times S + A \times T \times S_{M} - Ca^{45}_{R} \quad (4)$$

At sufficiently short intervals Ca^{45}_{R} can be disregarded From observations made at two different intervals T_1 and T_2 we then get

$$Ca^{45}_{1\text{-}Obs} = E \times S_1 + A \times T_1 \times S_{1\,M} \quad (4\ 1)$$

$$Ca^{45}_{2\text{-}Obs} = E \times S_2 + A \times T_2 \times S_{2\,M} \quad (4\ 2)$$

From these two equations we obtain

$$\frac{Ca^{45}_{1\text{-}Obs} - A \times T_1 \times S_{1\,M}}{Ca^{45}_{2\text{-}Obs} - A \times T_2 \times S_{2\text{-}M}} = \frac{S_1}{S_2} \quad (5)$$

After A has been calculated F can be obtained by introducing the calculated A value in equation (4 1)

In view of our previous discussion of the heterogeneity of processes and rates, it would be surprising if any one of these three terms could be positively linked to any single fraction function, or structure The authors do state for example that the exchangeable Ca of the skeleton consists of a [probably large] number of subfractions. However this may prove to be a helpful if necessarily simplified analysis of the many interrelated processes of isotope fixation, *if* the terms employed and the *limits of applicability of the method are clearly defined In this* method, *E* is a measure of the *reversible* exchange only The term *A* includes, besides new-crystal formation and crystal growth all irreversible processes (10)—the entrapment of isotope by recrystallization (Ca^{45}) intracrystalline exchange (Ca^{45}) and maturation (Ca^{45} and Na^{22}) The term *R* represents, in addition to resorption and remodeling any errors in the estimation of *E* and *A*

As we might expect then, there are important limitations to the

applications of this approach. For example it is stated that in the rat, *resorption, R, becomes significant in 16 hours (12)* yet *in the young* human it is stated that *R and E* can be ignored at 6 days (14). Further in the derivation given above, relation (2) is based on the assumption that $SA_E = SA_{blood}$ yet a recent publication (12) states that SA_E almost never equals SA_{blood}. In other words these workers are finding that if *A* is assumed constant (at short time intervals where *R* can be ignored) *E* is found to vary and conversely if *E were* assumed constant, *A* would vary. In one of the latest applications (14) it is assumed that $P^{32}_{Obs} = P^{32}_{A}$. To assume that a single rate process governs isotope fixation does not seem justified (10) regardless of the time interval chosen. If one is willing to accept simplification to this degree, it would be simpler to measure the total retention of an injected dose of radiocalcium over the 6-day period. This would obviate the necessity of a bone biopsy and would be more representative of the skeleton as a whole. We may expect interesting developments in this field. Perhaps electronic computers will aid in solving problems. In any event we can predict, from these interesting beginnings, that the kinetic approach will become more involved not further simplified.

REVIVAL OF HEVESY'S CONSTANT SPECIFIC ACTIVITY

Some of the complexities of the problem can be simplified if an old trick is employed. This trick was often used by Hevesy (48) who kept the *SA* of the blood "constant" by frequent injections of isotope. A modernized version is to feed animals a diet of constant *SA* for an extended period as first reported by Kon and associates (84). With this innovation the *SA* of the blood, the extracellular fluid, the exchangeable fraction of bone, the new mineralization and the mineralization in re-forming Haversian systems are all approximately equal after an initial equilibration period of 24 hours. The problems of interpretation are greatly simplified especially if adult animals are studied. This is not to say that the interpretations are simple, accurate or definitive, as will be seen.

Recently Stoll (80) employed the long lived radiosodium Na^{22} in diets of constant *SA* in an attempt to determine in the diaphyses of the young adult rat the magnitude and rates of some of the processes by which bone takes up this radioisotope. Since sodium is limited to the crystal surfaces, some of the processes defined at the beginning of this section—i.e. recrystallization, intracrystalline exchange, and crystal growth—are not operative and can be ignored, permitting a more ac-

curate delineation of the remaining processes. This is not possible when radiocalcium, radiostrontium, or radiophosphate is used.

In such an experiment, the *RSA* of bone, the ratio SA_{bone}/SA_{serum}, is governed as illustrated in Figure V 11 by the following processes:

A. Rapid exchange of extracellular radiosodium with sodium in the surfaces of the crystals of the available skeleton. The contribution of this process to the total *RSA* of the bone will be constant after an initial equilibration period and can be evaluated separately by extrapolating the observed curve of *RSA* versus time to zero.

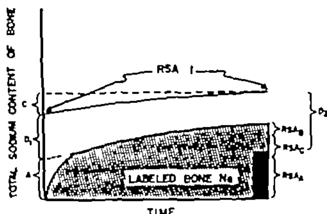


FIG. V 11.—A diagrammatic illustration of Stoll's analysis of data obtained by feeding rats diets of constant *SA* (radiosodium) for 3 months (see text for explanation of terms). The black bar represents the exchangeable sodium in control animals (not fed radiosodium) injected at the end of the experiment. The curve indicated as *RSA* = 1 is the total bone sodium which, if completely labeled, would give an observed *RSA* = 1.

B. Remodeling of established bone (in rats, primarily growth remodeling) with the replacement by new mineral of *RSA* = 1 of an equivalent amount of mineral of lower *RSA*. Unfortunately, we must "guesstimate" the *RSA* of the resorbed mineral. The *net* contribution of this process to the total *RSA* is given by the difference between the final, observed *RSA* and the contributions of processes A and C.

C. The net acquisition of new mineral. In the long bone diaphyses of rats of this age, more new mineral is added by new-crystal formation (*RSA* = 1) than is lost by resorption. The contribution of this process to the total *RSA* can be determined by the increase in ash weight of the bones during the experimental period.

It follows, then, that

$$RSA_F = RSA_A + RSA_B + RSA_C ,$$

where RSA is the ratio SA_{bone}/SA_{serum} and where the subscripts F, A, B and C refer to that finally observed after 3 months on the radioactive diet the sum of the fractional contributions of exchange of remodeling and of net acquisition, respectively With only one assumption that the Na/mineral does not significantly change (71) in old and new bone throughout the experimental period simple algebra gives us (80)

$$RSA_F = \left[\frac{W_I}{W_F} \times RSA_I\right] + RSA_B + \left[1 - \frac{W_I}{W_F}\right]$$

where RSA_F is the final observed RSA RSA_I is the value given by extrapolation of the RSA curve to time zero and W_I and W_F are initial and final weights of ashed bones. The RSA_B can thus be calculated.

But $RSA_B = RSA_{replaced} - RSA_{resorbed}$ where $RSA_{replaced} = 1$ The rate of turnover from remodeling therefore depends on an assumption as to the degree of labeling of the bone resorbed. A minimum rate is obtained if one assumes that the resorbed bone contained no radiosodium If one assumes that the resorbed crystals were of $RSA = 1$, the resorption rate could be infinite. The most reasonable assumption for these bone samples is that the resorbed bone had an over-all RSA equal to the average RSA of the whole diaphysis.

Stoll also determined the bone sodium unavailable for exchange at the end of the experiment by injecting control litter mates (not on the radioactive diets) with radiosodium and, after 24 hours, determining the value $D_2 = (1 - SA_{bone}/SA_{serum})(W_F/W_I)$ From this could be subtracted the bone sodium unavailable at the beginning of the experiment $D_1 = 1 - RSA_I$ to give D the *net* rate of maturation (total maturation rate minus rate of resorption of mature bone) or the rate of growth of the unavailable skeleton

There was a large standard error in Stoll s values, and in any difference calculation the errors of each term are shared with the others. Stoll s chief problem was concerned with sampling Because sodium has an extracellular distribution, it was necessary to use only marrow free shafts to reduce the contamination with non-skeletal sodium. This introduced a sampling error and at the same time, prevented a direct measurement of ash weight increase He was forced to deduce this increase from growth rate data obtained from other animals in the colony With Ca^{45} this problem is obviated but other problems are introduced The results of Stoll s analyses are assembled in Table V-3 The

observed percentage of bone sodium available for exchange agrees well with published data (cf Table V 5) The calculated rate of remodeling seems reasonable If the assumption that bone resorbed had an *RSA* equal to the over all average *RS* 1 is correct, about twice the net increment in bone growth is actually deposited and about half of this is resorbed again in remodeling

Two reports have appeared in which diets of constant calcium specific activity were employed That of Tomlin *et al* (85) in which it was found by quantitative radioautography that the radioactivity of cortical bone in the mid shaft regions (cf box in Fig V-8) increased almost linearly with time Extrapolation of the curve to zero time gave the percentage of readily exchangeable calcium as 0.2 per cent of the total calcium, and the slope of the curve gave a measure of the continuing mineralization and intracrystalline exchange, 1 per cent/week (remodeling in this area can be considered minimal) Weikel has reported (89) a preliminary study of whole femur using standard counting procedures for Ca[45] He also found an almost linear increase in the radioactivity of whole femur with time The readily exchangeable fraction by extrapolation was about 4 per cent and most of the radioactivity found in the femur after 68 days could be attributed to growth—the net acquisition of new mineral

TABLE V 3

PROCESSES OF ISOTOPE FIXATION IN DIAPHYSES OF LONG BONES OF RAT AS DEDUCED BY MEANS OF RADIOSODIUM (80)

	Per Cent Total Bone Sodium
Initially available sodium RSA_t A	51
Finally available sodium	45
Remodeling B	0 14/day*
	0 40/day†
Net acquisition of mineral C	0 31/day
Net maturation D	0 38/day
Av wt. of rats initially	180 gm
Av wt. of rats finally	220 gm.

* Assuming that resorbed bone contained no radiosodium

† Assuming that resorbed bone contained average *RSA* of whole diaphysis.

For the most part these three studies are quite comparable the major difference is, of course the percentage of the skeletal pool that is readily exchanged 51 per cent of shaft sodium 4 per cent of whole femur calcium and 0 2 per cent of mid-shaft calcium Much of these

analyses for sodium are also performed From the ratio SA_{bone}/SA_{plasma} the fraction of available bone sodium is obtained directly A number of such experiments have appeared in the recent literature, and the compiled results are given in Table V 5 A series of values obtained on rats of different ages is presented graphically in *Figure V 12*. These data, though scanty suggest that a minimum value of one-fourth to one-third of the bone is available in the adult animal, regardless of species. In the rat at least there is a regular decline (log-log plot) in the percentage of available skeleton with decreasing growth rate (increasing age)

TABLE V 5

COLLECTION OF MEASUREMENTS OF AVAILABLE SKELETON AS INDICATED BY SODIUM EXCHANGE

Species	Age	Total Bone Sodium Exchanged (Per Cent)	Crystal† Sodium Exchanged (Per Cent)	Ref
Man	11–67 yr	34		41
Man	8–67 yr	26		28 56
Man	40–65 yr		24	55
Man	Adult		35	34
Dog	Adult	44	41	34
Rat	Young adult (220 gm.)	51	36	11
Rat	Young (120 gm.)	55		40

Compiled by Dr G B Forbes.

† Total sodium minus sodium in bone water. This correction is based on the chloride content of bone on the assumption that the Na/Cl ratio in bone water is the same as in extracellular fluid.

The results with radiosodium are supported by other data. If we are permitted the reasonable assumption that the percentage of a dose of an injected isotope that is rapidly fixed by the skeleton is a reflection of the percentage of bone mineral available then there is good evidence in the literature that the available skeleton declines with increasing age. This has been observed with Sr^{89} and Ca^{45} (26) with $C^{14}O_3$ (22) with $P^{32}O_4$ (20), and UO_2^{++} (62)

The available skeleton expressed as a percentage of total bone weight may be misleading. For example Forbes (39) has found that, despite a *percentage* decrease the actual size of the available skeletal pool of sodium remains approximately constant (in mEq/kg bone) with increas-

ing age Perhaps this seeming paradox can be explained in part by the greater total amount of mineral in the adult animal and in part by the rate of Haversian remodeling which apparently is inversely proportional to rate of growth (76) If these interesting findings are substantiated we may conclude that there exists an approximately constant skeletal pool of available mineral throughout life

BUFFERING VERSUS REGULATION

In earlier chapters we discussed the variability inherent in the apatite crystal because of its small size large hydration shell and

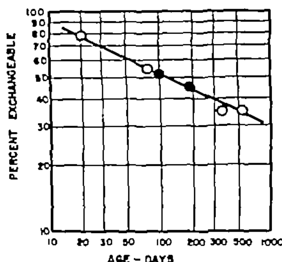


FIG X 12 —The decline of the available sodium of bone with increasing age of the rat. Open circles taken from Forbes (84) solid circles from Stoll (80)

adaptable crystal lattice Its composition therefore reflects, to a limited degree, the composition of the fluids which surround it. On this basis, normal bone composition is only a reflection of the normal electrolyte composition of the body fluids. Therefore, bone mineral cannot regulate electrolyte levels rather it merely resists their alteration. If the history of the individual has been normal the composition of the bones is normal and in the event of a sudden disturbance in electrolyte balance the bones will tend to return the body fluids to their normal composition. On the other hand if a chronic electrolyte disturbance which resulted in the laying-down of bone of abnormal composition is corrected, the bones will equilibrate with their new ion environment, thus resisting and delaying the return of circulating levels to normal com

position The skeleton, then by virtue of its large mineral content, acts as a non regulatory 'buffer' to changes in the electrolyte composition of the body fluids.

The bone mineral itself can be considered to be an antacid. Are not calcium phosphate preparations prescribed as such? In undergoing dissolution, the phosphate ion of the bone crystal changes from the trivalent state to a mixture of divalent and monovalent ions ($HPO_4^=$/$H_2PO_4^-$) To do this, the phosphate group must acquire hydrogen ions from the medium By means of mobilization of bone mineral, then, the animal can muster an enormous alkaline reserve with which to neutralize absorbed or metabolically produced acid. The converse is, of course, possible By transferring Ca^{++} and $PO_4^{\equiv}$ to bone, a great deal of acid (hydrogen ions) can be released Unfortunately, though we know that the skeleton can and undoubtedly does play an important role in acid-base regulation and conversely that acid-base conditions affect calcium and phosphate metabolism the magnitude, the mechanisms, and the controls of these interrelated events are not yet fully appreciated.

The passive non regulated response of the skeletal mineral must ultimately invoke regulatory responses. In sodium depletion, for example as sodium ions are drawn from bone, they must be replaced in part by calcium ions from serum Falling serum levels of calcium will stimulate the parathyroids to secrete. In other words, *no major shift in electrolytes can be postulated which would not affect, in some way the serum levels of calcium or phosphate Thus indirectly active homeostatic mechanisms are called upon*

TIME LIMITATIONS

Because of the continuing maturation of the structural elements of bone, the available osteones of today are the unavailable osteones of tomorrow This means, of course, that if a deficiency of a particular ion, say Mg^{++} is of prolonged duration, much of the matured unavailable bone will be abnormally low in Mg^{++} content. Even if Mg^{++} is restored to the diet, this matured bone will not show an increase in Mg^{++} content only the available skeleton can respond. Conversely, it is not possible by overloading to build a permanent labile reserve in the skeleton because of its ultimate burial in the unavailable structures.

This time limitation on the availability of skeletal depots has assumed an importance of enormous dimensions in our nuclear age. Many of the fission products, whether produced by peaceful power reactors or warlike bomb explosions, concentrate in the skeleton. Long-lived

strontium 90 is the most hazardous in this class of radioelements It would be highly desirable to develop methods by which these radioelements could be washed out of the skeleton Unfortunately, because of the continuing maturation process only bone structures formed in recent weeks are in contact with the circulating fluids. With passing time, the bulk of the radioactivity resides in the unavailable skeleton—out of reach of any conceivable therapy short of massive skeletal demineralization

It is this maturation of skeletal elements which explains the failure of chelating agents such as ethylenediaminetetra acetic acid (EDTA, Versene) and its congeners to mobilize radioelements from the skeleton These agents do indeed increase urinary excretion of skeletally deposited radioelements from a very very small percentage per day to a very small percentage per day But in terms of a reduction of the body burden, these agents have all proved to be resounding flops" (46), particularly if time has elapsed between exposure and therapy

EXAMPLES OF SKELETAL BUFFERING

It is only quite recently that the participation of the skeleton in electrolyte balance has been fully appreciated Therefore there is a very limited literature upon which to call to illustrate the preceding discussion with documented data

The rapidity with which the skeleton responds was illustrated some time ago by transfusion experiments which demonstrated that an amount of calcium three times that in the extracellular fluids was quickly mobilized from bone (47) Conversely it has been shown that the skeleton can remove from the circulation 27 mg of injected calcium within 15 minutes (24) The bone carbonate has been shown to be responsive even to short exposures to atmospheres of altered pCO_2 (42 67) A 20 per cent decrease in bone sodium and 12 per cent decrease in carbonate have been shown to result from a 4-hour intraperitoneal dialysis against ammonium chloride (17)

The non-regulatory nature of bone buffering is well illustrated in even the older literature In 1932 Irving and Chute (49) found that substantial amounts of carbonate were lost from the bones of rats and guinea pigs undergoing acute acid loads. More recently it has been shown that the PO_4/CO_2 ratio of the bone is a reflection of the PO_4/CO_2 ratio of the serum (77) An acute lowering of bone magnesium has been seen to occur in convulsive magnesium-deficient rats (69) All these

observations indicate that the composition of bone is not fixed but merely reflects the internal electrolyte balance.

The influence of maturation in rendering bone changes irreversible has been illustrated by Duckworth and associates. When magnesium was restored to the diet of depleted rats (33) the skeleton regained magnesium at a rate much slower than the original rate of liberation (32) Similarly, McCance (54) suggests that skeletal sodium can be drawn upon to remedy a shortage, but it is reported (79) that attempts to restore the 'lost sodium' of adrenal insufficiency failed to result in sodium deposition in the mid shaft of the femur of the dog The very fact that the excretion of a skeletally deposited radioisotope does not stabilize at a fixed percentage shortly after administration but continues to fall to ever decreasing values is perhaps the best evidence we have that more and more isotope is becoming buried with passing time (10) A good example of the importance of the mineral maturation processes is seen in rickets. In severe rickets the serum product, $[Ca^{++}]$ [total phosphate] falls to low levels. Pre-existing bone fails to continue to its full mineralization and newly synthesized osteoid and cartilage fail to initiate mineralization. In such a situation the available skeleton represents a greater proportion of the total than normally because of the failure of osteones to mature. Therefore a greater percentage of injected isotope is deposited initially than is observed in normal animals. This applies to Ca^{45} Sr^{89} (26), and UO_2^{++} (61) Despite greater deposition the radioisotopes are not retained. Because of the failure of normal accretion and mineral maturation, the isotopes are not buried irreversibly but continue to be reversibly washed from the skeleton by dilution with absorbed minerals and subsequent excretion (26)

The magnitude of ion transfers to and from the skeleton has not been extensively studied. Duckworth *et al.* (33) state that about a third of the skeletal magnesium can be mobilized in severe magnesium deficiency and Blaxton (19) adds that only the skeleton responds. Soft tissues show a remarkably constant magnesium content. Bergstrom found that no more than 29 per cent of the bone sodium content can be mobilized by any procedure studied thus far (18) He also calculated that bone provided 28 per cent of the total sodium lost during the depletion These values for sodium and magnesium are approaching in magnitude the proportion of the skeleton which we have seen is available Bone has been listed as one of the tissues to show a differential

rise in sodium concentration in response to sodium loading" (41) and Nichols and Nichols (66) have summarized results obtained in adults as follows 6 to 13 per cent [of skeletal sodium] can be mobilized in response to various stimuli Bone can also act as an acceptor of sodium ions, up to at least 8 per cent of the normal crystal content.

One of the problems in evaluating the magnitude of ion transfers to and from the skeleton is the contribution of growth If, in a depletion study in rats, the skeletal content of a particular ion—say, sodium—is decreased by 30 per cent what proportion of this decrease resulted from actual mobilization of sodium from *pre-existing* bone and what proportion resulted from the laying-down of new bone deficient in sodium? It is obvious that the study of young growing animals, rats in particular which may be adding to their skeleton at a rate of more than 10 per cent per day may amplify the apparent lability of the skeletal mineral depots.

SUMMARY

The three ion-transfer mechanisms characteristic of hydroxy apatite in vitro—(*a*) diffusion in the hydration shell (*b*) ion exchange at the crystal surface and (*c*) intracrystalline exchange—all occur in the animal but they are modified under the influence of physiological conditions new growth vascularity remodeling and maturation of bone and the regulation of absorption and excretion of minerals in the intestine and kidney At the histological level the age of the bone structure is the primary determinant of its reactivity The newly forming osteone very quickly mineralizes to nearly 90 per cent of its maximal mineral content displacing the water of hydration in the process and thus inhibiting the penetration of ions. At full mineralization, physicochemical reactions grind to a virtual halt Such old unreactive bone elements comprise the metabolically inert unavailable skeleton which in the adult animal is about two-thirds of the total

The continuing maturation of bone imparts an irreversibility to the exchange of ions, and, while injected crystal-seeking radioisotopes show a histological distribution reflecting the patterns of growth and remodeling, the complexity of the overlapping rates of exchange new growth, remodeling and maturation has frustrated attempts to analyze these processes quantitatively At present, it can only be said that the exchange process predominates in short term (24-hour) observations and that new growth remodeling and maturation dominate longer

periods of observation The maturation process is also responsible for the permanence of skeletally deposited body burdens of radioisotopes and the failure of therapeutic procedures to mobilize them.

There is, however a continuous supply of young reactive bone made available by growth and Haversian remodeling This constitutes a mineral reservoir which acts as a buffering system for such essential electrolytes as Na^+ $CO_3^=$, and Mg^{++} as well as participation in homeostatic control of Ca^{++} $PO_4^=$, and pH Major shifts in electrolyte balance must be presumed to invoke regulatory mechanisms which are poorly understood. The secretions of the parathyroid glands and vitamin D can be considered to have important functions in these regulatory mechanisms and are considered in detail in the following chapter

CHAPTER VI

Physiological Regulatory Mechanisms

We have discussed the participation of bone mineral in buffering against changes in the distribution of electrolytes in the circulating fluids. We have said that these events cannot be separated from the active homeostatic mechanisms which are working continuously to maintain normal levels of calcium and phosphate in blood It is now time to consider these homeostatic mechanisms, to describe, insofar as present information permits, the sites of regulation the regulating agents, and the underlying metabolic events involved The principal sites of regulation are three the portal of entry (intestine) the storehouse (bone) and the portal of exit (kidney principally perhaps also intestine) The principal agents are two vitamin D and the secretions of the parathyroid glands. The underlying metabolic events appear at the moment to be changes in carbohydrate metabolism in cells at the sites of regulation. Undoubtedly, as our knowledge increases, the more subtle effects of other tissues and other agents in the homeostasis of serum calcium and phosphate will be understood. For the present, the gaps in our information render it difficult to delineate the roles of even the principal participants.

One fact stands as a keystone to our understanding of homeostatic control. This fact was first developed in chapter II, where it was shown, on physicochemical grounds, that the *circulating fluids are normally supersaturated with respect to bone mineral* If the serum is supersaturated, then so also is the extracellular fluid. It follows, therefore that the fluid bathing the bone crystals must differ in composition from that of the extracellular fluids. We have seen that this difference lies not so much in the $a_{Ca^{++}}$ a_{HPO_4} as in the concentration of ions which tend to solubilize bone mineral so as to maintain the $a_{Ca^{++}}$ a_{HPO_4} at normal serum levels. Stated another way there is a gradient of specific ions which tends to lower the degree of supersaturation of the serum product, $a_{Ca^{++}}$ a_{HPO_4} As proof one can cite the rapid decline in the uct $a_{Ca^{++}}$ a_{HPO_4} in serum following parathyroidectomy or in *D* deficiency

The supersaturation of the circulating fluids imposes a similar problem on the intestine Some solubilizing mechanism must be postulated by which the intestine can transfer calcium and/or phosphate to the already supersaturated circulation. Similarly the kidney must actively resorb both calcium and phosphate ions, to prevent their loss to the urine

First to be considered are the secretions of the tiny parathyroid glands. These small bits of tissue by evolution, have come to dominate calcium and phosphate homeostasis so completely that they are necessary for health—even for life itself

Actions of Parathyroid Secretions

Much has been written on the nature the effects, and the sites of action of parathyroid secretions, though the chemical nature of the active principle(s) is still unknown. Dominating thought for many years was the concept (1) that parathyroid hormone caused increased urinary excretion of phosphate as its primary effect. Secondarily this *phosphaturia caused a hypophosphatemia thus inducing dissolution of* bone salt, which, in turn, raised serum calcium levels.

Gradually this older concept has given way to the idea of a dual action of parathyroid secretions. Currently it is believed that the principal and primary effect is on bone itself resulting in a transfer of calcium and phosphate from bone to serum. Secondarily and independently there is a decreased reabsorption (or active secretion) of phosphate in the kidney tubule leading to phosphaturia and hypophosphatemia. At least three authoritative reviews have appeared quite recently (5 48 53) summarizing the evidence for these conclusions. The transition in viewpoint has prompted McLean (53) and Howard (48) to speculate concerning the *mechanisms* involved in the transfer of calcium from bone to serum. Based on our physicochemical development of the blood-bone mineral relationship and new evidence for a metabolic action of parathyroid extracts we shall propose a possible mechanism.

Mechanism of Hypercalcemic Effect of Parathyroids

Both McLean and Howard though differing in details, developed the following thesis (*a*) removal of the parathyroid glands causes a sharp drop in serum calcium from a total of 10 mg per cent to 6 or 7 mg per cent (*b*) skeletal mineral maintains serum calcium at 6–7 mg per cent *without parathyroid intervention* (*c*) parathyroid activity then

causes bone mineral to support higher levels than expected from simple solubility considerations.

But how can a hormone affect a solubility equilibrium? McLean has this to say (53) "There is no way in which a solution of a salt, in contact with the solid phase, may become supersaturated by simple equilibrium with the solid. Supersaturation requires some sort of active intervention not merely the dissolving of the salt in the fluid" While Howard says (48) "Since many types of environmental changes, both local and general can alter the relationship between the skeletal crystals and the extracellular fluids, it is difficult to escape the concept that *this relationship is guarded or supervised by a living mechanism*"

It must be! How otherwise could it be that in the normal animal serum calcium remains one of "nature's physiologic constants" while serum inorganic phosphate varies with the absorptive state. We have said in chapter ii, on physicochemical grounds, that no K_{sp} exists for bone mineral, and here is the physiological confirmation Some cellular control of bone–extracellular fluid ion transfers must be operative But what is this "living mechanism" and how does it respond to the secretions of the parathyroid glands? McLean speaks of a "dual mechanism" referring to simple dissolution of bone salt and active cellular dissolution. Howard refers to a "membrane" or "barrier" or "gradient." The concept of a gradient resulting from cellular activity under hormonal control seems quite reasonable It is probably not necessary to postulate that a continuous membrane covers the bony elements even though such may exist—a gradient could be established without it.

In compact bone at least it has been shown that nearly all the water present can be attributed to the Haversian canals, the cells, and their canaliculi (chap v) This means that the ratio of cell volume to fluid volume is very high Would it not be easy for the cells to alter the fluid environment of the bone crystals bordering their canaliculi by metabolic or secretory activity? Inspection of Figure VI 1 is very reassuring Compact bone is thoroughly interlaced with interconnecting canaliculi Harris and Ham have emphasized recently (41) that, while these canaliculi permit the cells to survive and account for the transfer of nutriment and waste, they provide a rather poor system for diffusion throughout the bone which "requires that ... even in dense bone no cell is generally more than a small fraction of a millimetre from a capillary." This system of [illegible]

swamp water or bone fluid is determined not solely by the fast flowing stream but also by the soil and the life in the swamp itself or in this case, the mineral composition and metabolic activity of the cells. This relatively slow turnover of fluid in bone is readily seen in the blood disappearance curves of bone seeking isotopes discussed earlier (chap. v) The curves consisted of a series of exponentials of which the *third* rate term described in part at least isotope pickup by bone Transcapillary



FIG. VI 1 —Histological sections of bone showing the distribution of intercellular canaliculi (courtesy Amprino)

exchange and most of the extracellular mixing dominated the first and second rate terms. In view of the relatively slow fluid interchange and the ratio of high cell volume to fluid volume in bone, it seems reasonable to postulate that local conditions and concentrations differ somewhat from those expected of a simple ultrafiltrate of plasma.

How might cellular activity under the proposed conditions of relative isolation of bone fluid, alter the *apparent* solubility of the solid phase or decrease the degree of supersaturation of the serum $a_{Ca^{++}} a_{HPO_4^{=}}$? Since circulating serum is supersaturated with respect to bone mineral in vitro the net result of cellular activity must be to maintain a fluid composition in which bone mineral is more soluble or the serum

product $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ is just saturated Three mechanisms by which this might be accomplished seem reasonable

a) The presence of a local high concentration of a solubilizing ion As discussed in chapter ii certain ions which participate in surface exchange increase the apparent solubility of hydroxy apatite (Mg^{++}, $CO_3^{=}$ citrate$^{\equiv}$) or decrease the degree of saturation of a given product, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$

b) The production of a localized high concentration of a chelator of calcium Such a substance would reduce the concentration of calcium ions locally preventing the crystals from growing or actually causing the crystals to dissolve The calcium in chelate form would then diffuse into the venous circulation But only a small proportion of the calcium in the blood is in the form of a chelate (chap i) Therefore it must be postulated further that the chelating substance produced in bone is rapidly oxidized extraskeletally to leave free ionic calcium in the circulation One might also postulate an analogous mechanism for rendering phosphate non ionic A chelator seems unlikely but it would be possible to postulate a local synthesis of phosphate esters which would be hydrolyzed upon reaching the circulation.

c) The localized production of acid. Such an acid production would produce locally a pH lower than that of the general circulation Bone mineral is more soluble the lower the pH These slightly acid fluids, in diffusing back into the circulation would be brought back to pH 7 4 by the blood buffers. Again it must be postulated that extraskeletal oxidation of the acids produced in bone must occur Otherwise the blood buffers would soon be exhausted

That the secretion by bone cells of acids (especially citrate) would be a means of maintaining serum calcium levels has been suggested by Nordin (61) There has been a long history of speculation on whether in bone a local pH gradient might exist. Most of this speculation has concerned the induction of the *precipitation* of bone salt It was assumed that the serum was just saturated and that a locally elevated pH would cause precipitation. The postulate presented here is quite the reverse since we know serum to be supersaturated normally, it is necessary to propose that a locally depressed pH *prevents* the growth of bone crystals at the expense of the circulating fluids. The fundamental basis for this postulate is the inverse relation between pH and the solubility of hydroxy apatite which was described and illustrated in chapter ii Figure II 5

bone salt more soluble locally is the secretion of metabolically derived organic acids, citrate in particular Of all possible anions, citrate exerts a most powerful influence on the solubility of hydroxy apatite. This can be illustrated by the results from the study of a model system presented in Figure VI 2 (cf also Fig. II 7) It is seen that the solubility of apatite expressed as the product $a_{Ca^{++}}$ $a_{HPO_4^{=}}$ even after correction for chelation effects, rises sharply throughout the physiologi-

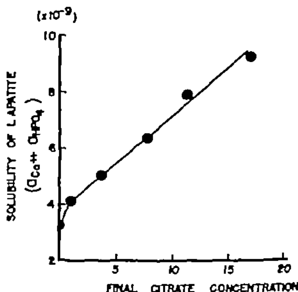


FIG VI 2.—The solubilizing action of citrate ion on hydroxy apatite. Taken from Strates and Martin (75) Conditions $\mu = 0.16$ pH = 7 4 $t° = 25°$ C., equilibration time 10 days solid-solution ratio = 5 gm/l, solid phase = L-apatite. The physiological range in serum is 3–5 mg. per cent; bone fluids may contain as much as 9 or more mg. per cent, as shown in subsequent sections. Actually in experiments in vitro such as these (3 75) the cause of the increased solubility is the large increase in $a_{HPO_4^{=}}$ as phosphate ions are displaced from the crystals by citrate ions (cf chap. iv)

cal range with increasing concentrations of citrate Also of possible chelating substances, citrate is ideal for our present purpose. It forms a highly associated complex with calcium (cf Table I 2) It is readily and rapidly oxidized by extraskeletal tissues (kidney in particular) In terms of acid production, citrate has three carboxyl groups, and all three are ionized at physiological pH Finally in terms of carbohydrate metabolism, citrate is the product most efficient in all three respects. A comparison with lactate for instance is shown in the accompanying

table. It must be presumed that parathyroid secretion induces the bone cells by synthesis to produce one or more of the following: solubilizing ions, chelating substances, or a lower environmental pH. To do this the hormone must alter metabolic processes by affecting some enzyme or enzyme system. This kind of postulate is generally sought as the mechanism of action of hormones, even though no hormone has yet been shown conclusively to act by specific regulation of an enzyme system.

	Lactate	Citrate
Effect on solid-phase solubility	Negligible?	Pronounced
Chelation	Negligible	Pronounced
Hydrogen ions produced/mole glucose	2	3

DIRECT EVIDENCE OF INDUCED CITRATE PRODUCTION

To our knowledge only one brief report (57) has appeared which *directly* supports the development just given. It is so brief that much can be *quoted here*:

It was hypothesized that cellular elements of bone normally secrete citrate (or citric acid) in response to parathyroid activity. This citrate carries complexed calcium to the serum where extraskeletal tissues (the kidney primarily) oxidize much of the citrate leaving an excess of calcium ion in solution. If citrate were secreted as the acid, a local pH gradient would also contribute to the transport of ionized calcium to serum.

It was technically very difficult to test the hypothesis directly. However, by simply drilling a small hole in the spongiosa of the femur of an intact dog it was possible to collect blood directly from the spongiosal circulation for comparison with simultaneous arterial samples. While this sample was only "contaminated" with venous flow from the bone cells, the output of citrate from the bone was so great, clearcut analytical differences were easily shown. Furthermore, dramatic increases in citrate output from the bone were observed almost immediately following injection of parathyroid extract. Simultaneous measurements of the clearance of intravenously administered radiostrontium gave a measure of the proportion of collected sample which was actually venous flow from bone. A typical experiment is summarized [in Fig. VI 3].

This direct substantiation of the initial hypothesis was given strong support by enzyme studies *in vitro*. It was found in confirmation of Dixon and Perkins (24) that mature bone lacks isocitric dehydrogenase which is needed for citrate utilization. Furthermore, parathyroid extract was shown spectrophotometrically to destroy the chromophoric group (340 mμ) of reduced

lactic, oxalacetic citric, isocitric and aconitic. Such a limited utilization of carbohydrate would be quite inefficient and one would predict a rapid depletion of cellular glycogen stores, as has been observed (46) Qualitatively similar effects would be expected from a *partial* destruction or inhibition of coenzyme II, but there would be quantitative variations in response from cell to cell depending on comparative enzyme coenzyme and substrate levels.

The morphologists have brought to light a question for which it is difficult to find a biochemical answer What changes an osteoblast into an osteocyte into an osteoclast? The rate of mitosis of the bone cells is

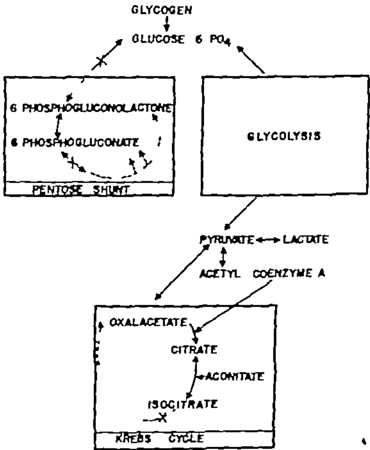


FIG VI-4.—A diagram showing how the blocking of coenzyme II reactions would block oxidation by the Krebs cycle and pentose shunt. *X* designates reactions requiring coenzyme II.

quite low yet a few hours after large doses of parathyroid hormone there is a large increase in the proportion of the bone cells which appear to be actively resorbing bone (45) Other thorny questions come to mind Why are bone cells so responsive to parathyroid hormone? Do other tissues respond similarly? If so why are bone and kidney target organs? Why do the histologists see in a single resorption cavity bone resorption and deposition occurring simultaneously and in close approximation? How can erosion and formation of Haversian systems occur simultaneously?

It is much too early to speculate on the answers to these questions. A large number of possible explanations come to mind and these are worth listing However there exists no experimental basis for choosing which may be likely which unlikely

Target Organ

This may be explained on the basis of (*a*) an unequal distribution of the hormone (unknown) (*b*) unequal permeabilities among organs (unknown) (*c*) unusual enzyme composition (low isocitrate dehydrogenase in bone and cartilage) or coenzyme levels or ratios of coenzymes (unknown) (*d*) unusual substrate relationships (kidney seems the principal site of oxidation of circulating citrate, bone may turn out to be the principal site of formation) and other less obvious variables, such as oxygen tension blood supply etc

Variability in Cell Response

On statistical grounds the individual cellular response can be expected to vary a great deal. At a given hormone level sensitive cells would be hard-hit, insensitive cells hardly affected. Statistics, however do not explain the organized activities of *groups* of cells, such as are seen in erosion cavities (all cells predominantly lytic) and in new forming Haversian systems (all cells predominantly synthetic) Here local factors, such as oxygen tension substrate supply pH, blood flow or unsuspected variables, must be operative For example we are not even certain that blood flow through a given Haversian blood vessel is continuous. Bone may be like the kidney in that only certain structural units, the nephrons (in bone the osteones) are active at any given moment If such were indeed the case, a nephron even momentarily without a blood supply would accumulate citric and other acids, with a resultant dissolution of mineral

Interconversion of Cell Types

It seems reasonable to suppose that the osteoblast is a healthy cell, well endowed with nutriment, oxygen and an optimal ionic environment and free from the stress of metabolic inhibitors. It also seems reasonable that under less optimal conditions, the osteoblast would lose first its specialized matrix-synthesizing functions conserving its activities to those required for the maintenance of its integrity as a cell. Under further duress, it would be expected ultimately to die, but in so doing would for a time pour metabolic acids into its environment and, unable to maintain its peripheral membrane, leak proteolytic and other enzymes and finally coalesce with dying cells. That parathyroid hormone as a metabolic inhibitor could markedly influence the distribution of bone cells among these three states of well-being seems clear It would be naive however to presume that this hormone is the only determinant involved

REVIEW OF LITERATURE FOR SUPPORTING EVIDENCE

Since Dickens first showed that bone contains relatively high amounts of citrate (20 21) there has been a continuing interest in this ion and its possible role in calcium metabolism. The literature documenting this interest has been reviewed for us quite recently (24 42) We need here only recount those papers dealing with the interrelations among parathyroid citrate calcium, and phosphate as outlined in our metabolic concept of parathyroid action.

Effect of Parathyroid on Calcium and Citrate of Blood

It has been repeatedly demonstrated that hyperparathyroidism (47) or parathyroid extract injection (2 52) increases serum citrate and that parathyroidectomy lowers serum citrate (26 27 33) We have confirmed these results in our own laboratory in dogs and rats. Admittedly this evidence is quite indirect but it certainly cannot be regarded as contradictory to our metabolic hypothesis.

More direct evidence has been brought forth by Freeman s laboratory In 1950 it was established that the kidney is responsible for the major share of the oxidation of circulating citrate in several species and that nephrectomy caused an elevation of the serum citrate and a transient hypercalcemia (31) A companion paper (32) demonstrated that the citrate and calcium increases following nephrectomy were dependent on normal thyroid parathyroid function Recently three papers (26

2, 33) have appeared which indicate quite conclusively that serum calcium, serum citrate, and parathyroid function are interrelated variables. The authors summarize (26) their results as follows:

1. Nephrectomy produced a transient hypercitricemia and hypercalcemia in the rat, rabbit, and guinea pig, but not in the baboon and monkey.
2. Parathyroidectomy 4 days prior to nephrectomy abolished the transient hypercitricemia and hypercalcemia that followed nephrectomy in the normal rat and rabbit.
3. Parathyroid extract restored the calcium and citric acid response of the parathyroidectomized rat to nephrectomy.
4. Vitamin D in amounts adequate to restore the plasma calcium values failed to restore the response of the parathyroid insufficient animal to nephrectomy.

While the authors state, "At the present time, there is no adequate basis for deciding what may be the source of circulating citrates," the discussion ends as follows: "The most plausible explanation seems to be that parathyroid hormone has some direct effect on citric acid metabolism." Actually, since parathyroid hormone has a direct action on bone *and* on citrate production, it is not unreasonable to presume that the increased citrate production occurred in the skeleton itself.

If, as our metabolic concept indicates, bone is a primary source of circulating citrate and if, among the possibilities suggested above, the response to parathyroid secretions might be inversely related to oxygen tension, it is interesting to learn that acute experimental anoxia causes a variable, though generally stimulating, response in circulating citrate (36). In some animals citrate levels were raised fourfold! Increased lactate levels were also noted.

Effect of Parathyroid on Citrate in Bone

It is important to recall that the citrate *content* of bone is necessarily a reflection of its *concentration* in the fluid bathing the bone crystals, since citrate exchanges for phosphate groups in the surfaces of the available crystals (cf. chap. IV). The citrate content of bone, then, is a more direct measure of the concentration of citrate in the bone fluids than it is a measurement of citrate in serum. One might expect that model systems in vitro would indicate what fluid concentrations are required to account for the citrate found in bone. Unfortunately, these data are not yet available.[1] It is reassuring, however, that Dickens

1. Armstrong's data (3) do not quite settle this crucial point. Bone gave up fair quantities of citrate at physiological concentrations of citrate when phosphate was

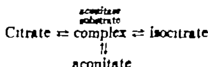
showed that prolonged administration of parathyroid extract to a puppy gave a 27 per cent increase in bone citrate (20) Since only the *available* skeleton could respond the actual rise in the citrate in the bone fluid must have been much greater than 27 per cent.

Carbohydrate Metabolism in Bone and Its Relation to Parathyroid Action

Thanks to Dixon and Perkins (23), we know that the enzymes necessary for citrate synthesis are present in bone and cartilage. They demonstrated the presence of citrogenase



and aconitase



On the other hand the enzyme necessary for citrate utilization—isocitric dehydrogenase—was deficient (unmeasurable in bone very low in cartilage) This would suggest that the citrate cycle does not function significantly in oxidation in bone cells, though Dixon and Perkins did not draw this conclusion. They did suggest that the citrate found in bone mineral could easily have been formed by the bone cells. Perkins and Dixon (65) also studied the effects of parathyroidectomy on rat bones. Citrogenase levels fell but slowly (4 weeks) while bone citrate levels did not change measurably The authors could not interpret these results neither can we.

A study (51) of the respiration of metaphyseal bone slices from *young rabbits suggests that the Krebs cycle in bone cells either* is not functioning or is operating at a low but maximal rate because of some rate limiting step (perhaps isocitrate dehydrogenase insufficiency) The authors did not draw this conclusion but they reported that the addition of glucose pyruvate, citrate, fumarate α-ketoglutarate, and malate did not affect respiration! With the exception of glucose all these acids, as members of the Krebs cycle, ordinarily act catalytically to increase oxygen consumption if the cycle is operative. Also of interest

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present in large excess when phosphate was absent, a little citrate was taken up. Similarly data obtained with a model system (75) give only an indication that bone contains more citrate than predicted from serum concentrations Additional work on this is needed.

and strongly in support of the metabolic concept as outlined above were their results obtained after injections of parathyroid extracts, 1 000 units over 42 hours. Endogenous, anaerobic glycolysis (lactate accumulation in N_2 atmosphere) was the same in both control and experimental slices. Unfortunately the rates of *aerobic* glycolysis were not studied Our metabolic concept predicts an increased aerobic glycolysis (defined as lactate accumulation) after parathyroid injection Respiration however was reduced to nearly half normal in the hormone-treated group The authors also report a loss in dehydrogenase activity " using succinic acid as a substrate This is difficult to interpret because succinate can serve as a hydrogen carrier quite apart from its role in the Krebs cycle In any event, the authors were prompted to suggest that perhaps some carbohydrate intermediate might accumulate under such circumstances, thus facilitating the transfer of bone salt to the circulation

Mineral Dissolution versus Matrix Attack

From the complexity of events involved in the deposition of isotopes in bone (chaps. iv and v) one might expect that very little could be added to our knowledge of parathyroid action by the use of Ca^{45} or radiophosphate However a recent report (82) gives quite conclusive evidence that parathyroid extracts induce the demineralization of *unavailable* skeleton. The success of this experiment rests on the time allowed to elapse between isotope injection and parathyroid treatment By waiting 72 days after the Ca^{45} administration these investigators insured that nearly all the remaining Ca^{45} was residing in fully mineralized, unavailable bone (4) The large increase in the excretion of Ca^{45} observed after parathyroid extracts could mean only a dissolution of that fully mineralized labeled bone Areas so fully mineralized that Ca^{45} could not diffuse in and out were suddenly rendered available to the circulation. Some change in the fluid environment adjacent to these unavailable crystals must have induced this dissolution. This evidence is quite convincing support for *a* metabolic concept of parathyroid action, though not necessarily the one outlined here. Certainly *some* cellular activity must be postulated to affect the bone fluid in such a way as to cause this extensive mineral dissolution

These results also cast doubt on suggestions that the primary action of parathyroid hormone might be the degradation of the bone matrix, which, in turn, "releases calcium (28) If radiosodium ions cannot penetrate the fully mineralized unavailable bone areas (chap v) how,

then could enzymes diffuse in to attack the matrix? At the present moment it seems best to conclude that at least a partial demineralization of the unavailable bone must precede any attack on the matrix. In our own laboratory hyaluronidase collagenase trypsin, chymotrypsin pancreatin and papain were all without measurable influence on powdered cortical bone *unless* a partial demineralization preceded enzymatic attack (56) Apparently in keeping with the extremely low content of water there are very few reactive groups (measured as free, e-amino groups) in intact bone (49) If parathyroid extracts mobilized calcium only from the not fully mineralized available bone then matrix attack might be considered a primary event. The Ca^{45} results, however show a parathyroid action on unavailable bone where demineralization must be a primary action

Finally there is some histological evidence which supports the metabolic concept One would expect to find the first signs of parathyroid action to be a dissolution of bone mineral around the osteocytes and their canaliculi Careful histological study (46) reveals, in the normal untreated animal "reactive bone mineral outlining the canaliculi and the osteocyte After injections of parathyroid extract the "reactive" zone around the osteocyte becomes enlarged, so that only the distal portions of the canaliculi are seen short and stubby but enlarged in diameter 'Reactive mineral in this case signifies staining with silver nitrate In the normal animal interstitial bone does not so stain Simultaneously with and corresponding to the pericellular loss of mineral, there appear positive signs of metachromasia of the matrix. This histological evidence is certainly consistent with a metabolic concept of parathyroid action.

Renal Effects of Parathyroid Secretion

That parathyroid secretion increases phosphate excretion by the kidney is an old observation but debate on this effect continues to the present (5) Is it a primary effect a secondary effect, or an artifact? Is it due to lack of resorption and/or secretion by the tubules? Certainly in the hyperparathyroid patient serum calcium is elevated, and serum phosphate is quite low conversely in hypoparathyroidism, serum phosphate is likely to be elevated. The phosphate clearance by the kidney has been shown to be high in hyperparathyroid states and low in hypoparathyroid states (5 60) Also in hyperparathyroid patients (60) calcium infusions (which presumably "shut off" the parathyroids) cause the phosphate clearance to fall while citrate infusions (presumed

to stimulate parathyroid secretion) cause the phosphate clearance to rise In hypoparathyroid patients, such infusions were without effect This kind of evidence may be more valuable than phosphaturic effects caused by various extracts " Since Stewart and Bowen showed that extracts of spleen and of thymus were capable of inducing phosphaturia (74) crude extracts are subject to suspicion. On the other hand it does not seem unreasonable to assume that the parathyroid hormone acts on more than one organ system and that the changes in kidney function following parathyroidectomy are quite real (76) Insulin does not act solely on the liver An inhibitor of cellular oxidation such as we have visualized might have a number of target organs. The kidney is the principal site of oxidation of circulating citrate If renal citrate oxidation were inhibited it seems reasonable to assume that an active process, such as tubular phosphate reabsorption might also be inhibited Unfortunately, this attractive speculation runs contrary to a growing body of evidence that the calcium mobilizing principle and the phosphaturic principle are separable (19 38 39 55) However all these reports base their conclusions on an assay ratio hypercalcemic/phosphaturic effects. Since degraded proteins can affect phosphate clearance and since the hypercalcemic effects are variable (cf next section) such evidence is not entirely convincing

Perhaps logic is our best weapon in lieu of critical information. Homeostatic control requires the operation of a kind of servomechanism A servomechanism requires a means of increasing *and* decreasing the variable under its control. In the present case the hormone in acting on bone can increase serum calcium levels only *indirectly* by raising the product $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ Actually as stated previously (chap IV also Fig VI 2) citrate production in bone would by displacement, tend to raise serum phosphate even more than serum calcium To obtain a truly effective rise in serum calcium, phosphate must be excreted The parathyroids, by ceasing their secretion, must also be able to lower serum calcium. The logical and available means is the elevation of phosphate in the serum If phosphate continued to pour out into the urine, the hypercalcemia would continue unabated even in the absence of parathyroid activity

On this basis, the parathyroids must exert a dual action (*a*) on bone they act to increase the product $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ in serum (*b*) on kidney they act to repress $a_{HPO_4^{=}}$ in serum Thus calcium is mobilized from bone to serum while phosphate is mobilized from bone to urine

THE HOMEOSTATIC CONTROL

From the viewpoint developed above it follows that the parathyroids influence calcium levels most effectively if phosphate levels are also influenced. Similarly the level of parathyroid secretion is influenced by the concentrations of both calcium and phosphate in serum (70). This results from the reciprocal relation between the two ions imposed by the solubility of bone mineral, *not* from a direct effect of the phosphate concentration on the parathyroid glands themselves. The bulk of the evidence (1 5 48 53) does indicate that parathyroid glands respond *directly* only to the concentration of ionized calcium in the serum and extracellular fluids. This is a reasonable conclusion. After all the activities of many cells, nerve and muscle in particular are very sensitive *to small changes in calcium ion concentration* (9). The fundamental basis of homeostatic control then is the relation

$$a_{Ca^{++}} \text{ in serum} = K \qquad (I)$$

where K is normally 0.5×10^{-3} (cf chap I) as set by the normal parathyroid condition. Secondarily phosphate is controlled because of the operation of the second fundamental relation

$$a_{Ca^{++}} \cdot a_{HPO_4^{=}} \text{ of serum} = R \qquad (II)$$

where R represents a range depending on the ion gradients between bone and serum normally $R = 1 \times 10^{-7}$. In the absence of gradients (parathyroidectomy) R appears to be about 0.6×10^{-7}. Under parathyroid stimulation R can reach a value of 2×10^{-7} or perhaps more. Thus we find that $[Ca^{++}]$ in serum is one of nature's physiological constants, while inorganic phosphate is somewhat variable. The limits of variability can be estimated by substituting relation (I) in relation (II) from which we obtain an $a_{HPO_4^{=}}$ in serum of from 0.12×10^{-3} to 0.4×10^{-3}. Converted to total inorganic phosphorus, this range is 1.9–6.5 mg per cent phosphorus, assuming a constant level of serum *calcium at 10 mg per cent.*

CONCLUDING THOUGHTS

This description of the homeostatic control by parathyroid secretions is derived largely from a liberal application of logic to a rather small core of established fact and clinical experience. Logic is indeed a powerful tool but it is no real substitute for experimentation. Unfortunately in our opinion the complicated effects of parathyroid secretion will not be finally settled until one or more pure chemically identi-

fied hormones are isolated from parathyroid tissue and the tissue "soups" or extracts of today are properly relegated to history.

When will the "soups" be displaced by pure components? Tomorrow perhaps. Perhaps not for years. The problem of purification has been rendered most difficult by the lack of a reliable and sensitive assay. Despite claims to the contrary, there is at present no satisfactorily reliable assay method. What function should be assayed, the hypercalcemic effect or the phosphaturic effect? The U.S.P. assay involves a serum calcium rise 18 hours after subcutaneous injection in dogs. Yet in rats the serum calcium falls to low levels 6 hours after parathyroidectomy (55). This suggests that the absorption of crude extracts is very slow. If absorption of a purified component were rapid, the standard U.S.P. test would give negative results with the very best fractions.

Why not inject intravenously? It is sometimes said that parathyroid extracts are ineffective when given intravenously. In our laboratory dogs, 1,000 units intravenously caused an initial small rise in serum calcium at 4 hours, followed by a slight drop at 18 hours, while a smaller dose, 60–90 units, gave good hypercalcemic effects (30) at 2 hours in some dogs and not in others. One could go on and on.

It all adds up to the fact that the literature cannot be evaluated critically. Quantitative comparisons are of doubtful significance. Negative results might be meaningless. Positive hypercalcemic activity is meaningful, but the time-dose response relation is too variable to assess quantitative relations accurately.

In view of all this, we are not really sure of anything. We are not even sure that the hormone is of a protein nature. It could be a carbohydrate, a lipid, or a complex of some kind.

Finally, the metabolic concept presented above, though attractive and in seeming harmony with much of the data currently available, is really quite tentative and insecure. It is presented only as a working hypothesis. Opinion which runs contrary to the hypothesis has been deliberately omitted, though contrary evidence was sought. Doubtless the concept is much too simple to be correct, yet it should prove helpful in designing future experiments.

Actions of Vitamin D

Today many vitamins have been assigned specific metabolic functions as coenzymes or coenzyme precursors. Yet, despite the fact that the major features of the D-deficiency syndrome were well outlined nearly fifty years ago and that vitamin D was the first vitamin to be

identified the mechanism of action of this vitamin remains an enigma. There persist uncertainties concerning even its gross physiological actions. Everyone agrees that vitamin D is necessary for normal intestinal absorption of calcium, but there is no unanimity concerning a direct skeletal action Everyone can understand how increased intestinal absorption favors elevated serum levels of calcium and phosphate and also how such elevated levels favor deposition of new bone mineral As a consequence many experts have felt that skeletal abnormalities seen in vitamin D deficiency are merely indirect consequences of the intestinal defect, and this view is difficult to refute experimentally Nonetheless, from the preceding discussion of the regulatory actions of the parathyroid hormone it should be clear that serum levels of calcium and phosphate cannot be either raised or lowered unless some alteration in the composition of the fluids bathing the bone crystals has occurred Indeed in the most recent literature, there has been a growing recognition that vitamin D seems to mobilize calcium and phosphate from bone to blood as well as from intestine to blood. This shift in emphasis is well illustrated in the writings of Nicolaysen and Eeg Larsen who in 1953 (58) stressed intestinal absorption and, three years later placed additional emphasis on the local action [on bone] of vitamin D (59)

DIRECT EFFECTS OF VITAMIN D ON BONE

Much of the recent evidence for a direct solubilizing action of vitamin D on bone has originated in Carlsson s laboratory This is a revival and extension of evidence and views now over twenty years old (69 70) Beginning in 1952 Carlsson demonstrated (10) that vitamin D raised serum calcium levels in rats on a diet almost devoid of calcium (0 04 per cent) In 1953 an extra intestinal effect of vitamin D was postulated (14) to explain Ca^{45}-retention data. In 1954 changes in the citrate content of bone and serum were found to be among the earliest effects of the vitamin when given to deficient animals (12) It was suggested that vitamin D may stimulate citrate production in bones and that this could be the cause of the increases in calcium and phosphate in the serum which paralleled those of citrate (12) The lowered serum product, $[Ca^{++}] \times [PO_4^{\equiv}]$ of D-deficiency was ascribed to a failure to mobilize stored bone salts (11) By varying the level of vitamin D dosage, intestinal and skeletal effects could be differentiated in rats. Maximal intestinal absorption was observed after only 10 I.U while bone salt mobilization was said to increase with doses up to 1 000 I.U (13)

Other laboratories have reported similar findings (70) Talmage and Dodds concluded that AT 10 (dihydrotachysterol) raised serum levels in parathyroidectomized animals by 'removing calcium and phosphate from bone (77) By manipulating levels of calcium in low-calcium rachitogenic diets Nicolaysen and Eeg Larsen found (59) that vitamin D treated rats exhibited higher serum calcium levels than did deficient rats, despite the fact that the deficient animals were absorbing more calcium! It is difficult indeed to explain such results exclusively in terms of intestinal absorption Vitamin D then not only improves intestinal absorption of calcium it seems also to increase the ion gradients in bone fluid resulting in higher products, $a_{Ca^{++}}$ $a_{HPO^{-}}$ in the circulation. Vitamin D may also have a direct action in promoting calcification (cf chap vii) but this gross action may be merely a secondary result of the elevated calcium-phosphate product

INTERRELATIONS OF VITAMIN D AND PARATHYROID ACTIONS

If vitamin D can mobilize calcium and phosphate from bone to blood, should we not consider the vitamin to be an integral part of the mechanisms responsible for normal calcium and phosphate homeostasis? Local dissolutions of bone mineral and the resulting transfer to serum is precisely the role we have assigned to the secretions of the parathyroid glands. Are the vitamin and the hormone interrelated in some way?

The idea of an interrelation between vitamin D and parathyroid hormone is, of course quite old. However the old concept proposed a direct stimulation of parathyroid secretion by vitamin D (8 34 54 64 68 78) which was easily disproved by the effectiveness of large doses of vitamin D in the *absence* of parathyroid glands. Justifiably this concept fell into disfavor in the early 1930 s (70) But we should not ignore all possible interrelations merely because *one* suggested interrelation has been disproved. Obviously the interrelation between the vitamin and the hormone, if a direct one exists, is complicated and subtle because the physiological actions of these two principles are in most cases quite dissimilar

Important differences are found in the histological changes seen in bone whether these changes are induced by excesses or deficiencies of either principle The pathology of hypervitaminosis D differs from the osteitis fibrosa observed in hyperparathyroidism the osteomalacia of rickets differs markedly from the pathology of hypoparathyroidism Perhaps the most striking differences in action of these two regulatory

principles are their extraskeletal effects. Vitamin D promotes the intestinal absorption of calcium parathyroid extracts may have such an effect but it has never been demonstrated. Parathyroid secretions appear to inhibit reabsorption (or stimulate secretion) of phosphate in the kidney Vitamin D if it has any direct renal action which is doubtful does just the opposite promoting phosphate reabsorption (44) and retention (70) except perhaps, in excessive dosage (17)

It is obvious that the vitamin and the hormone are different substances with different effects, acting at different sites. However in one respect these two principles have parallel or synergistic actions. Both raise the serum level of the product $a_{Ca^{++}} \cdot a_{HPO_4^=}$

This combination of differences and similarities is summarized in Table VI 1 where it is seen that the action of the parathyroids aggravates the rickets of D deficiency with high calcium diets (70) while they render rickets difficult to produce on low calcium diets, possibly by hyperplasia (37) Conversely vitamin D probably aggravates hypoparathyroidism with high phosphorus diets but helps to control the disease with high calcium diets (7) With proper dietary adjustments,

TABLE VI 1

A SIMPLIFIED COMPARISON OF ACTIONS OF VITAMIN D AND PARATHYROID HORMONE

	Effect on Serum by Action on		
	Intestine	Bone	Kidney
Vitamin D	↑Ca ↑P	↑$a_{Ca^{++}} \cdot a_{HPO_4^=}$	Negligible
Parathyroid	Negligible	↑$a_{Ca^{++}} \cdot a_{HPO_4^=}$	↓P

B DIETARY INFLUENCE ON SERUM REGULATION

Diet Ratio Ca/P	[illegible] Serum Ca or [illegible]		
	High	Normal	Low
D+Pth	n	n	n
Pth no D	↓P†	↓P	c
D no Pth	c	↓Ca	↓Ca‡

n indicates no change, normal, "c" indicates small change, near normal, compensated

† Accompanied by rachitic syndrome

‡ Accompanied by convulsive tetany

then each principle can compensate in part for a deficiency of the other The question immediately arises Do vitamin D and the secretions of the parathyroids raise the serum product a_{Ca} a_{HPO_4} by the same or similar mechanisms? Does vitamin D like parathyroid hormone induce citrate production in bone? Indeed it is well recognized that the citrate content of bone and serum is low in rickets and a rise in bone citrate is one of the earliest changes seen in the treatment of rickets with vitamin D (12 44 58) Two new questions come to mind Why does the parathyroid principle fail to induce citrate formation in D deficiency, and if citrate is not being produced in this condition how do the parathyroids so frequently maintain serum calcium at near normal levels in rickets? It appears that both vitamin D *and* parathyroid secretion are required for the normal production of citrate in bone. The metabolic effects of parathyroid though unsuccessful in producing citrate, cause some mobilization of calcium from bone to serum though less efficiently than normal

If we put all these considerations and facts into one over-all concept we arrive at the following analysis

Vitamin D and parathyroid hormone must act in bone at different sites in carbohydrate metabolism each with an independent effect so as to induce citrate formation synergistically Since we have proposed that the parathyroid hormone blocks the oxidative cycles and promotes glycolysis and the accumulation of pyruvic lactic and citric acids vitamin D must exert its action on some enzymic reaction between the beginning of glycolysis and the final condensation of pyruvate to give citrate. If vitamin D acts only on the condensation reaction and not on glycolytic reactions, the parathyroid hormone would still be capable of inducing pyruvic and lactic acid production and consequently some mobilization of bone mineral even in D-deficiency

PROPOSED METABOLIC ACTION OF VITAMIN D

It must be emphasized that in proposing a metabolic mechanism for the action of vitamin D that is related to a still hypothetical parathyroid action the authors are fully aware that they may be indulging merely in mental exercise It is fascinating however to fit together the following fragmentary bits of evidence to form an over all picture that makes some physiological sense

The first clue is provided by Tulpule and Patwardhan (80) who reported that cartilage in prolonged D-deficiency is unable to oxidize pyruvic acid added as a substrate The failure of D-deficient cartilage

to oxidize pyruvate is a most significant metabolic defect. According to current biochemical concepts, pyruvate is metabolized by conversion to citrate through the mediation of enzymes which require the following cofactors cocarboxylase (thiamin pyrophosphate TPP), lipoic acid (lipothiamide pyrophosphate LTPP) coenzyme A (CoA) and coenzyme I (diphosphopyridine nucleotide DPN) Thus

Pyruvate

(LTPP, CoA, DPN) (TPP)

acetyl CoA + oxalacetate → citrate

$O = C - COOH$, CH_3

CO_2 ← ← CO_2

$CoA - S - C(=O) - CH_3$ + $O = C(-COOH) - CH_2 - COOH$ $\xrightarrow{HOH}$ $CoASH + HO - C(COOH)(CH_2COOH) - CH_2 - COOH$

Oxalacetate is also derived from the citrate cycle but acetyl CoA is derived from pyruvate. Omitted in the diagram of the latter reaction are the phosphorylation steps which require ATP

The second report to be considered is the as yet unconfirmed and controversial paper by Zetterström and Ljunggren (83) wherein it was shown that phosphorylated vitamin D_2 activated oxygen uptake when added to kidney mitrochondria metabolizing glutamate as a substrate. Glutamate, upon oxidative deamination yields α ketoglutaric acid. Significantly the next metabolic step is the oxidative decarboxylation of α ketoglutarate a reaction thought to involve coenzyme A, coenzyme I, cocarboxylase and/or lipoic acid as cofactors (63) Is vitamin D involved in the synthesis or activation of one of these cofactors?

It may be more than coincidence that Räihä and Forsander (67) have reported a stimulating effect of vitamin D on the phosphorylation of thiamin These authors studied only the circulating levels of cocarboxylase (thiamin pyrophosphate) after injections of thiamin and vitamin D Unquestionably a real effect of vitamin D was observed but how plasma levels of cocarboxylase relate to tissue levels particularly in bone remains to be established Despite this uncertainty it is significant that the thiamin pyrophosphate in cocarboxylase and lipoic acid is required for the formation from pyruvate of both oxalacetate and acetyl CoA

The pyruvate to-citrate reaction has been shown in cartilage to be sensitive to the dietary level of vitamin D Dikshit Joshi, and Pat

TABLE VI 2

STIMULATION OF CITRATE PRODUCTION IN CARTILAGE BY VITAMIN *D* (22)

	Citrate Production (γ Citrate/Gm Fresh Cartilage)	
Controls (deficient)	18 ± 7	23 ± 6
Treated (4,000 I U)	47 ± 12	52 ± 6
	(24 hr)	(96 hr)

The authors termed the [illegible] of the tissue content of the condensing enzyme system, citrogenase The over all reaction in citrate formation was acetate + oxalacetate and ATP → citrate + ADP

wardhan (22) have studied D-deficient rats and demonstrated a sharp rise in citrate production within 24 hours after administration of the vitamin (an enormous dose 4 000 I U /rat) The salient findings are summarized in Table VI 2. The authors concluded that the increased citrate production was evidence that the concentration of the condensing enzyme citrogenase had increased suddenly in response to vitamin D administration This, of course is a possible explanation. However the speed with which the change occurred makes one wonder In general enzyme levels shift rather slowly to most physiological stimuli. An example of the case in point is the work of Perkins and Dixon which showed that citrogenase levels declined slowly over a 4 week period after parathyroidectomy (65) A more attractive interpretation, then would be that a deficiency in a required cofactor such as cocarboxylase or lipoic acid was catalytically relieved by the administra

tion of D In this connection it is highly significant that the incubating solutions employed by these authors contained Mg^{++} ATP cysteine etc., CoA and cocarboxylase were not incorporated. However, the interpretation of these results is not straightforward. Tissue slices, not homogenates, were studied. Since it is doubtful that ATP can penetrate intact cells and since no control experiments were reported (omitting ATP) these results, though significant are somewhat unclear

If vitamin D is required for the activation of the decarboxylation of pyruvate or one of the several reactions by which pyruvate enters the

TABLE VI 3

EFFECT OF VITAMIN D ON CARTILAGE METABOLISM FROM PICARD (66)

Cartilage	Mineralizing Solution (Micromoles /Cc)			Appearance in Medium (γ/10 M Cartilage)	
	Ca	PO₄	ATP	Lactate	Citrate
Normal	2 5 2 5	0 1 66	0 83 0	66 46	60
Rachitic	2 5 2 5 2 5	0 1 66 1 66	0 83 0 0 83	124 100 64 80 100	30 44 47, 47 41
Rachitic + 0 5 γ calciferol in medium	2 5 2 5 2 5	0 1 66 1 66	0 83 0 0 83	125 100 125	140 118 122

Krebs cycle, pyruvate and lactate would be expected to accumulate in D-deficiency Citrate levels, on the other hand, would fall. Picard has reported (66) interesting data on just this point lactate and citrate production by rachitic cartilage slices. Though somewhat preliminary in nature this study lends support to the previous development. His results are summarized in Table VI 3 Compared to normal, D-deficient cartilage produces more lactate and less citrate The addition of calciferol had no marked effect on lactate output, but it increased citrate formation by 200–300 per cent

Such a catalytic role of vitamin D could hardly be expected to be limited to a single tissue however Would not citrate synthesis in organs other than bone and cartilage be affected to some degree by variations in vitamin D intake? As if to reassure us, Steenbock and Bellin

(72) have reported that the administration of calciferol to deficient animals increases the citrate content of all organs examined with the exception of the liver They studied blood bone heart kidney intestine and liver

In developing this story" of the metabolic action of vitamin D certain publications were selected from many The scientific literature is to the would be theoretician like the Bible to the theologian By the selection of certain passages out of context a case can be made for almost any viewpoint. In this case where an attempt has been made to select the critical step in a complex cyclic series of events, the margin for error is very great. At the present time our knowledge of the metabolism of cartilage cells is inadequate (35) The metabolism of bone cells has been examined even less thoroughly There are some indications that there may be a deficiency of Krebs-cycle enzymes in both tissues (cf above) For any tissue the enzymes and cofactors participating in the decarboxylation of pyruvate and a ketoglutarate are not fully elucidated (63) We cannot predict in any detail therefore how vitamin D might promote or activate these reactions. Nonetheless with the background furnished by these six papers, it is easy to see how the actions of the parathyroids and vitamin D *could* be metabolically interrelated In D-deficiency falling blood levels of calcium stimulate parathyroid secretion The hormone, exerting its inhibition of oxidation (through its proposed action on reduced coenzyme II) causes the cells of bone and cartilage to glycolyze. The resulting accumulation of pyruvate and lactate cannot be converted to citrate because of the lack of phosphorylated thiamin or lipoic acid or other vital cofactors. Thus citrate levels in bone fall, despite the hyperactivity of the parathyroid glands. Conversely in the absence of the parathyroids, most of the carbohydrate would be oxidized through the pentose shunt, with little accumulation of pyruvate. Thus vitamin D which promotes conversion of pyruvate to citrate would be less effective than normal. In this scheme, illustrated in Figure VI 5 the parathyroid secretions shunt carbohydrate metabolism to the production of pyruvate and lactate vitamin D favors the production of citrate from these two acids, and the parathyroid secretion then inhibits the oxidation of citrate. Both parathyroid hormone and vitamin D can be expected to act independently of each other However the absence of either factor renders the other much less efficient and normal levels of citrate in bone can be expected only when the diet contains vitamin D *and* when the parathyroids are intact.

ACTIONS OF VITAMIN D ON CALCIUM ABSORPTION

We have stressed the action of vitamin D on bone yet the importance of this vitamin in maintaining normal calcium absorption is of paramount importance Does the metabolic concept, which explains the course of events in bone prove equally effective in explaining the vitamin s effect on the intestine?

The problem in absorbing calcium from the intestine is, in many ways, analogous to the problem of mobilizing calcium from bone. By whatever mechanism the mucosal cells absorb calcium and phosphate, an efficient absorption cannot be obtained unless the two ions are in soluble form. We have seen earlier that the solubility of precipitates of

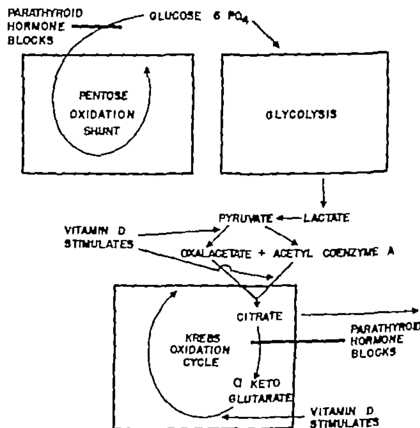


FIG. VI 5 —The proposed synergistic production of citrate by vitamin D and parathyroid hormone. Parathyroid hormone, by blocking oxidation cycles, promotes acid accumulation from glycolysis. Vitamin D by promoting more rapid conversion of pyruvate to citrate, favors the accumulation of citrate.

calcium phosphate is very low under physiological conditions of pH and ionic strength we have seen too that serum is supersaturated in this respect What is to prevent precipitated calcium phosphate from passing through the intestine without dissolving? In fact why is there not a constant drain from the body as the calcium and phosphate ions secreted into the gut in the digestive fluids are entrapped by the insoluble calcium phosphates in the intestinal lumen? It seems reasonable to suppose that as in bone some cellular induced gradient normally renders the intestinal contents favorable for the dissolution of calcium phosphate precipitates. As with bone the most likely mechanisms would be solubilization by exchange chelation and local acid production. Certainly anything that promotes an acid condition in the intestine will increase the solubility of calcium phosphates and favor their absorption. Similarly high concentrations of citrate or any other chelator of calcium will also provide a favorable gradient for transfer of both calcium and phosphate to the portal circulation.

Perhaps it is no coincidence that orally administered citrate is antirachitic (71) That its effects are indeed local is indicated by the observation that parenterally administered citrate does not cure rickets (60) If vitamin D in promoting citrate formation increased the citrate content of the intestinal mucosa or its secretions its action on calcium absorption might be accounted for As in bone, we would again have an operation of a citrate gradient by which calcium could be transferred to the circulation The threefold action of citrate—solubilization complexing and depression of pH—would be operative in the gut. The resultant elevation of serum calcium concentration would then deactivate the parathyroid glands lowering the citrate gradients *in bone* and thereby would promote skeletal deposition. Unfortunately despite considerable interest in calcium-citrate interrelations, few data bearing directly on this problem are available. The increase in the citrate content of intestine following administration of vitamin D to deficient animals, mentioned above (72) was not impressive but the entire intestine, mucosa *and* musculature, was analyzed Preliminary analyses of intestinal contents, too failed to show a significant amount of citrate (12 43) Many crucial experiments to test this point remain to be done but the problem of the role of citrate in intestinal absorption may prove difficult to elucidate. Under some circumstances, dietary citrate has been shown to be rachitogenic (16) and sodium citrate is less effective than some amino acids in promoting Ca^{++} absorption (81) In these two

studies there is some question concerning comparisons of dietary additives when the diets also vary in their acid-base balance

Very little is known of the metabolism of the intestinal mucosa, and, without such knowledge it is not possible to evaluate properly the metabolic concepts derived above for the actions of vitamin D If the intestinal mucosa, like bone, lacks a complement of enzymes required for normal operation of the Krebs cycle vitamin D might markedly augment citrate production At least, the metabolic concept suggests a number of pertinent experiments. For example one unusual prediction suggested by the metabolic concept relates to the importance of thiamin If vitamin D is somehow responsible for the activation of reactions requiring phosphorylated thiamin one should expect that this vitamin, too would be important in calcium absorption and metabolism It is true that the primary defect in thiamin deficiency does not involve mineral metabolism but rather nerve degeneration Nevertheless, it is quite encouraging to find a number of papers (6 18 25 50) all of which claim to show that thiamin increases the absorption and retention of calcium This relationship between thiamin and vitamin D though somewhat obscure at the moment deserves intensive study

There has been some indication that vitamin D lowers the pH of the *intestinal fluids and of the feces (73 84) However it is difficult to de*duce whether this is a *cause* or a *result* of improved absorption of calcium and phosphate. Unabsorbed calcium phosphate can be expected to render the intestinal contents more alkaline it is an alkaline salt. Anything that favors its absorption will automatically render the intestinal contents less alkaline Under these circumstances, which is cart and which is horse cannot be easily decided.

It seems reasonable to postulate the existence of a calcium 'pump in the intestine whatever the mechanism may finally prove to be. After all the amount of calcium transferred from the gut to the circulation seems to vary within surprisingly narrow limits, irrespective of the calcium content of the diet, from a little less than ½ gm. to perhaps 1½ gm. in the human [2] It gives one the impression that the intestine possesses a mechanism of very limited capacity to absorb calcium and that operation of this mechanism at a rate near to capacity is required for the maintenance of normal skeletal physiology At the present time, there seems to be no reason to postulate an intestinal pump" for phos-

2. Even when there is no *net* absorption from the diet, several hundred milligrams of calcium derived from fluids secreted into the gastrointestinal tract must be absorbed

phate (58) though the existence of an active absorption process for this ion cannot be denied

CONCLUDING THOUGHTS

As with the metabolic concept of the action of parathyroid secretions, the scheme proposed here for the physiological actions of vitamin D is intended as a working hypothesis to aid in the design of future experimentation The authors are only too painfully aware of the meager experimental foundation upon which a rather cumbersome theory has been constructed However the complex interrelated system described for the regulation of serum calcium by vitamin D and parathyroid secretions is consistent with present information and is inherently logical In these respects the system is reminiscent of the Albright Reifenstein concept of parathyroid action. Their scheme was consistent with data then available and was also logical. Regulation was presumed to result from renal induced phosphaturia calcium was affected only indirectly In the present discussion emphasis has been placed on the regulation of calcium, while phosphate regulation has been left dangling so to speak. Actually, this is more apparent than real In the present scheme the dual action of parathyroid secretions on bone *and* kidney provides for simultaneous regulation of both calcium and phosphate though calcium is the more rigorously controlled Thus, acting on bone, the parathyroids mobilize both calcium and phosphate to serum by stimulating citrate production. The kidney effect however is to reduce serum phosphate by inducing phosphate excretion. The net effect, then is a transfer of phosphate from bone to urine of calcium from bone to serum. Despite all that has been said concerning the lack of a fixed K_{sp} for bone mineral, in the absence of cells there is, as we have seen a reciprocal relation between calcium and phosphate in a solution in contact with bone mineral or hydroxy apatite On this basis, elevated serum phosphate levels repress serum calcium activating the parathyroids to induce phosphaturia Conversely falling serum phosphate levels permit greater amounts of calcium to dissolve from normal bone and the resulting hypercalcemia turns off' the parathyroids, inducing phosphate retention There is only one commonly observed phenomenon which seems contradictory When one infuses calcium, serum phosphate rises. Since this rise occurs even if parathyroid extract is given with the calcium infusion (62) it cannot be attributed to renal retention. Nor can we attribute this rise to dissolution of bone for it violates all chemical experience. Phosphate should be deposited in bone during

calcium infusions. The only explanation in the authors opinion, must be given in terms of an extraskeletal cellular response. Do high calcium levels induce cells to lose phosphate? There is no adequate experimental evidence upon which to base an answer at present.

The metabolic concept of vitamin D action presented here suggests that vitamin D might have direct action on bone cells, which would favor mineral deposition. If the vitamin is a part of or is required for the synthesis of a cofactor needed in the decarboxylation of α-keto acids, *its presence would promote efficient cellular oxidation of carbo*hydrate its absence would make the cell more dependent on glycolysis. But glycolysis is synonymous with acid production and the accumulation of acids renders the normal product $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ less supersaturated. By inhibition of glycolysis, vitamin D would promote mineral deposition.

Another puzzling problem may be explained in terms of the blood-bone gradient, namely the demineralization that occurs in immobilization. For a given level of parathyroid hormone and vitamin D we should expect a fairly steady production of citrate in bone. With a fixed production however the *gradient* of citrate will be inversely proportional to the rate of blood flow A sluggish circulation will leave the bone cells steeped in accumulating metabolic, acid end-products, resulting in a demineralization of the affected limb

CHAPTER VII

Mechanisms of Calcification

In chapter i it was established that the activity product, $a_{Ca^{++}} \cdot a_{HPO_4^{=}}$ of normal serum hovers around a mean value of about 1×10^{-7} In chapter ii, it was established that this mean value is both undersaturated and supersaturated at one and the same time. This paradox was, of course easily resolved in chemical terms. We are dealing with two different solids (*a*) the solid phase which precipitates initially is secondary calcium phosphate (*b*) the only solid phase which is stable at physiological pH is hydroxy apatite Normal serum then is undersaturated with respect to secondary calcium phosphate but supersaturated with respect to hydroxy apatite. This is all very clear and easily understood. Physiologically however the problem remains paradoxical In the formed bone the cells are faced with the problem of being literally engulfed by a surfeit of calcium and phosphate. They must actively resist the pressure of the supersaturated fluids, which tends to increase mineralization to the exclusion of water and the inhibition of homeostatic ion transfers. At the same time in areas of forming bone and cartilage, the cells after laying down matrix are faced with the problem of completing their task—mineralizing the matrix they are supplied with fluids containing calcium and phosphate in concentrations now insufficient to induce precipitation of *new* crystals (precipitation of $CaHPO_4$ with subsequent hydrolysis)

This is the heart of the problem. Even though the circulating fluids are supersaturated with respect to bone mineral, new crystals do not form spontaneously How is this accomplished in growing bone and cartilage? Evolutionary processes could have selected one of two possible mechanisms to induce new-crystal formation The first is obvious. A booster mechanism could be developed by which local concentrations of calcium and phosphate are raised above the critical ion product needed for precipitation The second possible mechanism is much less obvious. Based on the principle of *epitaxy* or seeding rather than on precipitation, a crystalline substance (possibly organic in nature) may

be elaborated This material, by virtue of its crystalline structure, which is sufficiently similar to the structure of hydroxy apatite, is able to induce the aggregation of a nucleus of calcium and phosphate ions. Given a crystal nucleus of hydroxy apatite, the body fluids are sufficiently supersaturated to form a complete crystal spontaneously

The differences between these two possible mechanisms may be illustrated by analogy (Fig. VII 1) In Figure VII 1 *A*, we have pictured

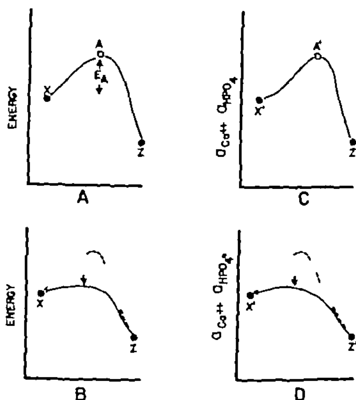


FIG VII 1 —The analogy between the catalysis of chemical reactions and the catalysis of crystal formation (see text for explanation)

the conversion of compound *X* to compound *Z* a reaction which is energetically feasible because of the lower energy content of the product *Z* In practice, however we find that the reaction does not proceed spontaneously We must put energy into the system (E_A the energy of activation) to bring compound *X* to an activated state, whereupon it converts to *Z* more or less spontaneously We can however avoid the input of energy by the introduction of a catalyst, as in Figure VII 1 *B* In this case the catalyst (in biology the enzyme) activates *Y* permit

ting the spontaneous conversion to *Z*. By analogy, the product $a_C \cdot a_{HPO}$, X, of serum is sufficiently higher than the dissolution solubility of hydroxy apatite *Z*, so that this solid should form spontaneously. In practice, however, we find that the reaction does not occur spontaneously. We must, as in Figure VII 1 *C*, put calcium or phosphate into the system to bring it to Y, the minimum product for spontaneous precipitation, the $K_{sp\,HPO}$ (the "activated state"). This would require a booster mechanism. We might avoid raising the activity product by the introduction of a surface catalyst, as in Figure VII 1 *D*, which induces the aggregation of ions to form a crystal nucleus. The formation of hydroxy apatite *Z* would then proceed spontaneously with little or no increase in the product $a_C \cdot a_{HPO}$, X, normally found in the body fluids.

The former, uncatalyzed, reaction we shall term the *booster mechanism*. The latter, catalyzed, reaction will be termed *epitaxy, seeding mechanism, or crystal nucleation*.

Energetically, the seeding mechanism is the more economical and efficient. Control of rates of crystallization and orientation of the crystals which form is implicit in crystal seeding but difficult to arrange in an outright precipitation of $CaHPO_4$ and its hydrolysis to apatite. The organic portion—collagen fibers and polysaccharide—is a necessary part of the over all bone structure. If some part of this organic framework can induce crystal-seeding, then crystal growth will occur spontaneously, since the fluids are somewhat supersaturated. However, to induce precipitation by a booster mechanism, large extracellular gradients of calcium and/or phosphate must be postulated. In pure inorganic solutions at 37° C, an ion product three times that of normal serum is required. In the presence of the solubilizing foreign ions and the crystal poisons of the extracellular fluids, the required ion product would be even higher.

On teleological grounds, therefore, we should expect the mineralization process to be a biologically controlled crystal seeding, wherein crystal nuclei are formed as an end product of cellular activity. Historically, however, the investigators in this field have almost invariably attempted to demonstrate the presence of a booster mechanism in areas of mineralization.

The Robison Scheme

The reason the literature is a record of persistent devotion to a single approach is that Robison's phosphatase theory (43) dominated re-

work (24 43 45) However there are three pertinent observations in the literature which must be explained before any alternative proposal can be expected to displace the Robison scheme in the minds of men These are the following

A The invariable presence of phosphatase at the site of mineralization, as first shown by Robison We might ask why phosphatase is found in non mineralizing areas but this is hardly an adequate answer to the question "What is it doing in the mineralizing areas?

B The improved calcification in vitro of slices of epiphyseal cartilage from rachitic rats seen when equivalent concentrations of ester phosphate are substituted for inorganic phosphate in the fluid medium

C The close correlation between calcification in vitro of rachitic cartilage slices and glycolysis and its phosphate ester intermediates.

Later on when we have built a frame of reference we shall discuss more fully these three findings which we may regard as the heart of the case for Robison s theory Now we must turn to the crystallographers for the rules governing crystal induction and crystal-seeding These rules are of interest even if one subscribes to some modified form of Robison s phosphatase theory

Epitaxy

Royer in 1928 introduced the term epitaxy' to describe the phenomenon of crystalline intergrowth or oriented overgrowth (44) From Greek, this term translated literally means on arrangement " Today epitaxy is a branch of mineralogy with thousands of examples of oriented overgrowths that have been analyzed structurally No general quantitative theory has yet been evolved but the field has been systematically reviewed for us by Seifert (46)

When a drop of a hot sodium nitrate solution is allowed to cool on a freshly cleaned surface of calcite sodium nitrate crystals form on the calcite surface and are oriented so that the crystallographic directions of sodium nitrate are parallel to the corresponding directions in the calcite crystals. These two crystal types are isomorphous. Epitaxy is not however confined to isomorphous crystals. For example, fresh cleavage faces of mica will orient NaI KCl, KBr KI RbCl, RbBr RbI NH_4Cl, NH_4Br and NH_4I but not the remaining alkali halides. Cubic KI shows epitaxy on hexagonal calcite. Even $K_2Zn(CN)_4$ is oriented on mica. The examples run into the tens of thousands.

Royer (44) concluded that any two of the following three conditions must be satisfied for an induced orientation

1 The lattice spacings (or the distances between two ions) in the two crystal faces in contact must be almost identical or be in a simple numerical ratio the lattice spacings in the other directions are irrelevant

2 Ions, which in the growing crystals are situated along an extension of a row of say x ions in the host crystal must be of the same sign as are the x ions.

3 Chemical bonding of crystal elements must be identical in the two crystals.

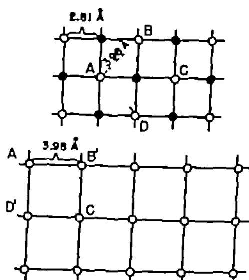


FIG VII 2.—The epitaxy of NH_4Br on NaCl after Royer (44)

Of these three the first condition is the most important. An example in Figure VII 2 illustrates this point. In the presence of urea, NH_4Br crystallizes on NaCl with its cube edge parallel to the diagonal of the cube face of the NaCl In the NH_4Br lattice, the distance between two ammonium (or two bromine) ions is 3 98 A. In the NaCl lattice two sodium (or chloride) ions are separated by 5 63 A but parallel to the diagonal this distance is only 3 98 A, i.e. $(\sqrt{2}/2) \times 5.63 = 3.98$ A The NH_4Br crystals thus assume an orientation in which the ammonium ions 'match the positions of the underlying sodium network.

The match between the lattice of the forming crystal and the host crystal need not be exact, but the degree of permissible tolerance is not known with certainty One mathematical discussion (19) predicts a critical value of misfit of 9 per cent above which epitaxy becomes less

likely Up to a misfit of 14 per cent the possibility of epitaxy exists if the temperature is relatively low Above this value the forming crystal embryos dislocate spontaneously and while oriented overgrowth will not occur there still exists the possibility that the host crystal may provide for the growth of crystal nuclei These nuclei would then seed the supersaturated solution giving unoriented crystal growth

Where the lattice constants are well matched crystals of one substance may seed a supersaturated solution of another substance very efficiently This is the property of epitaxy which interests us most We have said that the body fluids are supersaturated with respect to calcium and phosphate. Yet we must explain why crystals normally form only in the *right* places. Epitactic induction of oriented crystal nuclei is an elegant mechanism by which to meet nature's needs

Epitaxy of Hydroxy Apatite

We know that hydroxy apatite and bone mineral can serve as their own host crystals in epitaxy Thus if such crystals are added to a stable but supersaturated solution of calcium and phosphate ions, new-crystal formation is induced This is illustrated with bone by the following simple experiment (38) Twenty milligram samples of bone ashed in KOH and glycol were suspended in a solution the pH and composition of which corresponded closely with an ultrafiltrate of normal serum This solution by itself, was stable for months. After the suspended ash samples had been agitated for 24 hours, they were centrifuged the supernatant solutions aspirated for analysis, and fresh ultrafiltrate added to the ash residues. This process was repeated daily for 17 days. The results are illustrated in Figure VII 3

The ash removed calcium and phosphate every day for 17 days. The amount of solid phase was increased by 50 per cent and exhibited a molar calcium to-phosphate ratio of about 1 5 While in this particular experiment it was not established whether the crystals grew or new crystals formed the latter seems most likely As discussed in chapter II the crystals of hydroxy apatite do not grow beyond colloidal dimensions.

Brushite crystals ($CaHPO_4 \cdot 2H_2O$) can also induce apatite formation if the pH is high enough This is probably not properly classified as epitaxy however At neutral or alkaline pH brushite crystals disintegrate, and the disintegrating pieces form hydroxy apatite (52)

These two examples of seeding of hydroxy apatite crystals, though interesting are hardly applicable to our basic problem—mineralization

in the animal In the cartilage and in new osteoid, crystals must be induced to form in an area where no crystals pre-exist. If epitaxy is the mechanism of calcification the host crystal must be organic, not inorganic, in nature

The only crystalline material of organic composition yet described to be present in cartilage *and* bone is, of course collagen This protein is crystalline in its native state and gives an X ray diffraction pattern more or less characteristic of all collagens. Here is a most interesting analogy to the hydroxy apatite systems. In chapter iii we found the main stumbling block to the understanding of the crystallography of

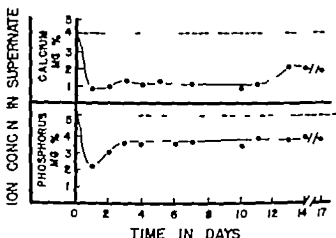


FIG VII 3 —The induction of hydroxy apatite formation by bone ash placed daily in a synthetic ultrafiltrate of serum. Dotted lines indicate the composition of the original solution.

apatite to be the awkward fact that a family of solids of varying composition exhibits the same structure by X-ray analysis. The various collagens, too vary in composition (amino acids) yet all exhibit the same *structure*

Can collagen produce an epitactic growth of hydroxy apatite crystals? The weight of evidence indicates that it can For example data assembled in Table VII 1 (52) show that while gelatin and fibrin were unsuccessful, reconstituted collagen fibers or apatite crystals could induce crystal formation from otherwise stable solutions of calcium and phosphate ions. All three protein preparations had been exhaustively dialyzed against versene and proved to be free of inorganic ions as checked by analysis for calcium and the collagen fibers had been twice

recrystallized (23). It must be admitted that these solutions were much more supersaturated than serum normally is, but it must also be recognized that the collagen fibers were derived from a tissue which does not normally mineralize. Two questions immediately arise: (*a*) Do collagen fibers derived from different sources vary in their ability to induce mineralization? and (*b*) Is it the bare collagen molecule, or is it something associated with the molecule, that is responsible for the nucleation of crystals?

TABLE VIII

CRYSTAL-SEEDING OF CALCIUM PHOSPHATE MIXTURES (29)

Compound Added	Amount (M[illegible] C[illegible])	Final Concentration			Degree of Crystallization
		Liquid Phase		Solid Phase M[illegible] (C[illegible]/P)	
		P M × 10	C M × 10		
None (control)		33.2	20.0		
Apatite	3.0	20.6	1.49	1.47	7
Apatite	3.0	21.0	1.01	1.75	8
Gelatin	3.0	34.0	19.5		
Fibrin	3.0	32.6	20.2		
Collagen†	3.0	29.6	13.0	1.43	1

Solutions buffered at pH 7.4. Mixtures equilibrated 10 days at 25° C, filtered through molecular filters, and ashed. Initial calcium and phosphorus concentration the same in all solutions and just below point of spontaneous precipitation.

† Reconstituted fibers (23) from tendon.

We have attempted in our own laboratory to prepare reconstituted collagen from skin, tendon, and bone. Good yields were obtained from skin and tendon, but the collagen derived from bone failed to dissolve in the usual reagents. It is possible that this is a reflection of fundamental chemical differences in collagen from different sources. It is also possible that during the *long* demineralization process (using approximately neutral solutions of versene) some essential mucopolysaccharide was lost. The presence of a mucopolysaccharide is apparently necessary for good crystallization of the fibers (22). This failure to obtain reconstituted collagen fibers from bone leaves the first question unanswered and underscores the second question. It is doubtful that crystals of collagen have ever been obtained which did not contain some polysaccharide (31).

At the present time, then, we cannot be sure whether collagen itself

or some molecule structurally associated with collagen (mucopolysaccharide) is responsible for crystal induction There is preliminary evidence that demineralized bone sections will remineralize in serum or serum-like solutions to give an apparently normal orientation of crystals (29) This may mean that in its native state the organic osteoid is actively epitactic in solutions having the same degree of supersaturation as normal serum.

It is of interest therefore, to examine the crystal structure of collagen for spacings that might correspond to the spacings of the apatite lattice This information is summarized in Table VII 2. Indexing the

TABLE VII 2

COMPARISON OF X RAY REFLECTIONS OF COLLAGEN (12 31) AND APATITE

INDEX OF AXIAL REFLECT (COLLAGEN)	SPACINGS IN FIBER DIRECTION		SPACINGS TRANSVERSE TO FIBER DIRECTION	
	Collagen (A)	Apatite (A)	Collagen (A)	Apatite (A)
0	20		10 4 11 6	9 88
2	9 55		5 [illegible]	
4	5 0	6 88	4 6	
5	3 97			
7	2 86–3 26	3 44		

reflections of collagen in terms of a simple unit cell is by no means straightforward the only clearly apparent layer line corresponds to an axial spacing of 9 55 A but neither of the prominent meridional arcs (axial spacings at 2 86 and 3 97 A) will fit a unit cell of 9.55 A. Some investigators feel that an axial period of about 20 A gives the best indexing

As can be seen, there is no exact correspondence in the spacings of the two crystals, but some are tantalizingly close A 20 A axial repeat in collagen is very close to three times the unit-cell distance (*c*-axis) of apatite 20 6 A. It has been found that the meridional reflection 2 86 A which is perhaps the most reliable and most characteristic of collagen is increased in the *stretched* state to 3.26 A (12)—very close to correspondence with the column calcium positions in apatite, 3 44 A apart The transverse reflection of collagen varies with the state of hydration—11 6 A hydrated and 10 4 A dehydrated. Caglioti (4 5) was probably the first to point out the possible relation between the

collagen and the apatite structures and while no final statement can be made at the present time it appears that the collagen structure is close both axially and transversely to the structure of apatite It is certainly conceivable (since the periods have been observed to be variable) that a minor structural alteration could transform a fiber of collagen just outside the tolerance limits for epitaxy to one within the limits of tolerance in *both directions—a* and *c* axes—of the apatite structure

This is the crux of the problem in its present stage of development (*a*) Can cells secrete protofibrils of collagen that aggregate spontaneously into fibrils which are active in the epitaxy of hydroxy apatite crystals? (*b*) Do the collagen fibers act secondarily by orienting a mucopolysaccharide in such a structural way that it becomes epitactic? (*c*) Do the complex fibers require some activation mechanism such as phosphorylation to render them epitactic? (*d*) Is epitaxy only an incidental event subsequent to a true precipitation as in some of the schemes based on the Robison theory?

There are no final answers to these questions. In fact there may be more than one mineralization mechanism Certainly as we shall show there are many differences between the mineralization of cartilage and that of osteoid

Structural Interrelations between Organic and Inorganic Constituents

If epitaxy were indeed the principal mechanism in the mineralization process, then we should expect to find co-orientation of organic and inorganic constituents in the bones and teeth Beginning in 1926 Funaoka (21) demonstrated preferred orientation of the crystallites in enamel. Thereafter Clark (11) showed fiber orientation in the long bones, and Stühler (53) found that the *c*-axes of the mineral crystals paralleled the fiber direction. Recently the close relation between the fiber direction and the crystallographic *c*-axis of the mineral has been demonstrated conclusively at the ultramicroscopic level (17 54) The most comprehensive discussion of crystal fiber interrelations has been given by Robinson and his associates (41 42 54) A summary of their recent views (41) may be quoted

> The collagen fibers of bone as observed after versene decalcification osmic acid fixation and plastic imbedding increase in size as the bone tissue matures. The five smaller band regions in each major period of collagen fibers, observed in newly formed bone give way to two-band regions in more mature bone. The two bands are described as a doublet " The inorganic component

of bone is deposited in the band regions of the collagen At a "calcification front" very small inorganic deposits about 20 Å. to 50 Å. are seen. It is our impression that these deposits are associated with the small period bands. Bits of other inorganic material of a more obviously crystalline form, are observed They are about 200 Å. to 300 Å. long and about 20 Å. thick. They parallel the long collagen axis and appear to be definitely associated with the doublet band areas that recur about every 640 Å. along the fiber axis. In general the smaller crystal and collagen sizes are associated with a more hydrated and less fully calcified matrix. The less distinct electron diffraction pattern obtained on recently calcified bone matrix suggests that the crystals are not only smaller, but that some of the observed inorganic components in newly formed bone may not be in a crystalline form. It is pointed out that both tanning agents and the bone crystals are associated with the band regions of collagen It seems likely that some of the inorganic component is inside the collagen fibers at the band regions as well as on the periphery of the fibers and in the interfibrillar areas. Electron micrographs suggest this position for some of the inorganic material in the fibers.

The intimate structural interrelations between the crystals and the collagen fibers in bone are strongly suggestive of active epitaxy However the structural situation in cartilage seems to be vastly different

Calcification in cartilage appears after the degeneration of the cells, whereas in bone it occurs close upon the laying down of the collagenous matrix by osteoblasts. Secondly the crystals are laid down at a distance from the edge of the cell capsule in cartilage but within a fraction of a micron of the bone cells.

This delayed potential for calcification is further emphasized by the space between the level of cartilage matrix formation and the level of its calcification. Thirdly the nidus of calcification appears to be on or closely related to the fibers in osteoid This may also be true in cartilage, but it is not demonstrable. The initial crystal arrangement in bone at the epiphyseal line is as irregular as that in cartilage but the crystals in the osteoid soon take on a pattern of orientation in relation to the collagen fibers. This coalignment of inorganic crystals and fibers is not observed at any stage of cartilage matrix calcification. The fibers of osteoid are 2 to 5 times the diameter of those in cartilage and have an obvious periodic structure not seen in the cartilage fibers.

Another difference in the organization of the osteoid and epiphyseal cartilage matrix is the packing of the fibers. Those in cartilage are widely separated before and after calcification but there is a marked change in the arrangement of the osteoid fibers a short time after crystal deposition starts.

From a volume standpoint, in the cartilage matrix, the space between the fibers is greater than that occupied by the fibers in osteoid within 2 to 3

> micra of the osteoid last the reverse situation obtains. Since this is the space in which the cement substance is believed to be located there is reason to presume that there is a greater volume of cement substance in a unit volume of epiphyseal cartilage than in an equal volume of osteoid.
>
> In previous studies on bone (42) it was suggested that although the bulk of the inorganic crystals was between the fibers there may have been some mineral in the fibers at the band region. It becomes obvious from these investigations that in epiphyseal cartilage the crystal deposits occur primarily *between* the fibers in the cement substance space. Using microradiograms Owen *et al.* (39) found calcified epiphyseal cartilage remnants in the diaphyseal sections of long bones of the rabbit. The calcified cartilage had more calcium mass per unit volume than even the most completely calcified bone tissue and therefore was distinguishable because of its greater absorption of x rays. The explanation would appear to be that there is more space for crystal deposition between the fibers in cartilage matrix than there is between the fibers in an equal volume of osteoid.

These two quotations from the work of Robinson and Cameron are presented as support of collagen-crystal interrelations. The two statements could be presented to support just as well a polysaccharide crystal interrelation. In osteoid much of the ground substance is associated with the fibers and probably therefore oriented with respect to the fibers, as the crystals are; in cartilage there is more ground-substance "space" which is not oriented with respect to the fibers but which nonetheless appears to be the location of the unoriented crystal formation.

Mineralization of Cartilage and Bone in Vitro[1]

In view of Robinson and Cameron's conclusions, it is advisable to consider the mineralization of cartilage as possibly different from the mineralization of osteoid. While the case for fiber-crystal epitaxy in bone is strong, in cartilage the connection between the organic and the inorganic components is much less clear. From the electron micrographs it would appear that, from single, isolated, embryonic crystals or nuclei, mineralization of the interfibrillar space occurs as a self-seeding, self-perpetuating process with little regard for the arrangement of the protein fibrils.

Despite the obvious resemblance of this process to an uncontrolled precipitation, there are still good reasons for believing that epitactic

[1] Omitted from this section are the studies on the significance of glycolysis. This important research, principally by Gutman and by Shorr and their associates, is discussed in the next section.

seeding is operative. The principal reason is, of course, that properly preserved non vital sections of rachitic cartilage will mineralize normally in vitro when placed in solutions below the point of spontaneous precipitation.

Once crystal nuclei have formed the cartilage will mineralize spontaneously even after much abuse. This was shown by Sobel (49) and DiStefano (14). The work from these two laboratories ascribes a paradoxical lability and stability of the nucleating mechanism in rachitic cartilage. The respiration of cartilage slices rapidly declines after removal from the animal. If the slice is in a non-mineralizing or non-protective medium there is a rapid loss of calcifiability, presumably due to autolysis (14). On the other hand, calcifiability can be "protected" by placing the slice in a solution of $CaCl_2$ (49) or of ester PO_4 or in a calcifying medium for a short period (14). Thereafter the slice will mineralize in the presence of metabolic inhibitors, after long periods of cold storage or other abusive tactics certain to insure cell destruction (14, 49). The paradox may be explained as follows. If the cellular integrity of the slice is not maintained, irreversible autolytic destruction of the seeding sites promptly occurs. If crystal nuclei have formed, however, or if the site is protected by combination with calcium ions, self-seeding will take place irrespective of the condition of the cartilage cells.

These results and the reversible, competitive inhibition of the calcifying mechanism by cationic substances substituting for calcium ions (26, 50) strongly suggest that the active nucleating site is anionic and combines with cations. Sobel feels that this kind of evidence implicates chondroitin sulfate as a component of the mechanism (50). Two studies of the ion-binding properties of chondroitin sulfate from hyaline cartilage demonstrated (1, 20) that divalent cations are preferentially bound by this mucopolysaccharide, but it failed to induce crystal formation at physiological concentrations of calcium and phosphate ions (1). From this we may conclude that if Sobel is correct in assigning chondroitin sulfate a place in the actual seeding mechanism, the chondroitin sulfate of mineralizing cartilage must differ structurally or in its combination with collagen from the chondroitin sulfate of non-mineralizing hyaline cartilage.

Concurring with Sobel that the mineralizing site is anionic, DiStefano *et al.* (16) have suggested a phosphorylated polysaccharide as a possible nucleation site, based on the chromatographic isolation of a substance having the properties of an amino sugar-phosphate ester

Its presence in cartilage was shown to correlate with the ability of cartilage to mineralize in vitro. Later the force of this suggestion was weakened by the demonstration of a widespread distribution of this still uncharacterized ester in various non-mineralizing tissues of the rat (51).

Another view of the seeding process in cartilage has been given by Cartier and Picard. Charging that hypertrophic rachitic cartilage is not "normal," these investigators chose embryonic material for study. The validity of this charge may be challenged on the grounds that rachitic cartilage will mineralize if placed in normal serum (55), while embryonic cartilage does not mineralize at comparable levels of Ca and $PO_4^=$. The difference may lie in the fact that most of the embryonic cartilage is not yet ready to mineralize—i.e., the seeding sites are not yet prepared—a process these workers must accomplish in vitro before mineralization takes place. Rachitic cartilage may not be a "normal" tissue but the seeding mechanism seems to be present and ready to function normally. Nonetheless, any argument concerning the normalcy of rachitic cartilage is somewhat irrelevant. Embryonic cartilage is good material for study in any event.

The development of this work is reminiscent of Robison's theory to the extent that it begins with a clear concept but grows in complexity with further study. Initially, they reported very poor mineralization of cartilage taken from lamb embryos when the calcifying medium contained inorganic phosphate or hexose phosphates, but very heavy mineralization if adenosine triphosphate (ATP) was added to the medium (6). Next they demonstrated the presence of a non-extractable enzyme, ATPase, differing from alkaline phosphatase in histological distribution, which had a pH maximum of 8 and was activated by magnesium ions (7). It was then shown that, under the influence of this enzyme, ATP was converted to adenosine monophosphate (AMP) and the pyrophosphate appeared "fixed" in the tissue in association with calcium (8). The phosphate deposited in the slice was estimated as 80 per cent pyrophosphate and 20 per cent orthophosphate. Next an attempt was made to demonstrate the presence of pyrophosphate in new formed bone mineral, but, if present, it was undetectable by the methods used (estimated <1 per cent of total phosphate) (9). This led the authors to presume that ATP is used only in the *initial* phases of mineral formation. Most recently, a publication appeared (10) in which glucose, hexose phosphates, and various inhibitors were tested both on mineralization and on glycolysis in vitro. The results were very compli-

cated and confusing. For example the presence of glucose (one-sixth to one-third of normal serum levels) markedly inhibited the mineralization caused by ATP It is impossible at the present time to pass judgment on the possible significance of ATP in mineralization processes.

Our present knowledge then, of the calcification mechanism of cartilage is quite meager We do not know whether the seeding mechanism is inherent in the collagen or in the carbohydrate fraction whether the enzymatic phosphorylation of the matrix is a required step or whether some other yet unnamed trigger mechanism is operative. The role of the cells is not clear Actually, the cartilage cells may *prevent* mineralization. If the cartilage cells secrete citrate and other metabolic acids as bone cells do, one would expect mineralization to take place only with difficulty whether a seeding template is or is not present In this connection it is important to know whether mineralization occurs in the vicinity of hypertrophic cells which are rich in glycogen or in the vicinity of the hypertrophic cells just after the glycogen has disappeared. It is a difficult point to settle (18) but there is some evidence for the latter view (36) Rarely does mineralization occur near young maturing cartilage cells. On the other hand the cartilage cells may *induce* mineralization by "activating" a seeding site on already secreted matrix. Finally it is possible that the cells do not actively participate in the mineralization process per se. Their sole contribution may be the secretion of calcifiable matrix.

Studies on mineralization of bone osteoid in vitro are practically nonexistent Here where the case for epitaxy of the inorganic crystals by collagen is strong on structural grounds, we have advanced very little since the earliest suggestions of protein-crystal interaction (5) Howard *et al* (29) recently showed that histological sections of trabecular bone could be demineralized by versene at pH 7 4 These demineralized sections (presumably non-vital) remineralize spontaneously in vitro to reproduce their original histological state. In our laboratory demineralization of bone sections at alkaline pH 9–10 destroyed the nucleating mechanism. This procedure is certain to extract mucopolysaccharides to some extent, but we cannot be sure that the collagen structure remains unaltered under these conditions.

Modifications of Robison's Scheme

Role of Phosphatase

As stated earlier the Robison theory is being supplanted by the theory of crystal epitaxy or crystal-seeding Yet a role must be assigned to

phosphatase and certain awkward facts must be explained before the ghost of Robison's theory is properly laid

In recent years an important function has been assigned to phosphatase a function which explains its occurrence in tissues other than bone and ossifying cartilage It is suggested that phosphatase is involved in the synthesis of extracellular matrix On histochemical and chemical grounds, a number of investigators have related phosphatase to the formation of fibrous protein (2 3 30 40) to matrix formation (47) to mucopolysaccharide synthesis (32) and to a transphosphorylation of some phosphate acceptor in the matrix (8 25) Any one of these tasks would constitute an adequate explanation for the presence of phosphatase in areas of mineralization but yet another possibility has been suggested Granting that the hydrolysis of ester phosphate is beneficial to crystal formation what about the converse? Are unhydrolyzed esters harmful? That the answer is an emphatic Yes is suggested by the commercial use of organic or inorganic polyphosphates to prevent boiler scale production. The added phosphates act like crystal poisons, preventing the formation of $CaCO_3$ crystals. That this is not a removal of Ca^{++} by chelation is shown by the fact that relatively insignificant quantities of phosphate are required in relation to the amount of Ca^{++} present Moreover direct tests have shown that a representative ester a glycerophosphate chemisorbs on hydroxy apatite crystals, inhibiting the usual induction of new-crystal formation from calcium phosphate solutions. This ester will also poison the mineralization of rachitic rat cartilage in vitro if phosphatase is first inhibited (15) This view suggests that the hydrolysis of ester phosphate at mineralizing sites is important not by locally increasing inorganic phosphate (negligible) but rather by removing a crystal poison from the immediate fluid environment (only negligible quantities are necessary for this function)

ROLE OF ESTER PHOSPHATES

Why then do ester phosphates give better mineralization of rachitic cartilage than do equivalent amounts of inorganic phosphates? How can a crystal poison be beneficial when present in high concentrations? No unequivocal answer can be given at the present time. Even Robison's original proposal does not explain this strange finding There is, however an explanation. When rachitic cartilage is sliced and placed in inorganic solutions, considerable quantities of phosphatase are leeched from the tissue and can be demonstrated in the medium If it is assumed that the crystal-seeding site is a phosphorylated compound

(consistent with Sobel *et al* [50] with DiStefano *et al* [16], and with Cartier and Picard [8]) it would be in danger of attack by the now solubilized enzyme The addition of ester phosphates to the medium would provide a competitive substrate to protect the seeding site from attack. Once crystal nuclei have formed the seeding centers are either protected or not needed. With passing time the phosphatase ultimately hydrolyzes the ester phosphate completely and its inhibitory action on crystal growth is stopped. In this view the presence of ester phosphates is both beneficial and harmful beneficial in the early periods by preventing autolytic destruction of the seeding site and *only temporarily harmful* by its action as a crystal poison All this may seem like far fetched speculation but it was demonstrated that the uncharacterized amino sugar-phosphate ester is destroyed when rachitic cartilage slices are incubated in saline but is stable in the presence of a medium containing ester phosphate (16)

How many of these problems arise from the artificiality of the in vitro experiment and how many are pertinent to physiological events are important but unresolved questions

ROLE OF GLYCOLYSIS

There remains the intimate connection between the mineralization of cartilage and glycolysis. First suspected by Creighton in 1896 (13) and later incorporated into the "second mechanism" by Robison this interrelation was given a firm experimental base by Gutman and associates (24 25) and later extended by Shorr and co-workers (27 28, 34 36) The gist of this relationship may be summarized thus

a) Glycogen which accumulates in cartilage cells, disappears just prior to or simultaneously with mineralization.

b) If an inhibitor of glycolysis is added to the mineralizing medium (containing *either* inorganic phosphate or glycolysis intermediates above the inhibited reaction) mineralization does not occur

c) If a phosphorylated glycolysis intermediate *below* the inhibited reaction is added to the medium mineralization of the cartilage slice occurs.

Because of these findings, Gutman (25) has suggested a modification of the Robison theory Instead of functioning as a hydrolytic agent to furnish inorganic orthophosphate phosphatase, according to Gutman, may serve in the enzymic transfer of a phosphate group from a metabolic ester to an acceptor in the matrix. This suggestion bridges beauti-

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